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Chelation, transport and physiological roles of iron in Aquaspirillum magnetotacticum

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Chelation, transport and physiological roles of iron in *Aquaspirillum magnetotacticum*

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CHELATION, TRANSPORT AND PHYSIOLOGICAL ROLES OF IRON IN AQUASPIRILLUM MAGNETOTACTICUM.

BY

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"They were the best years of my life," he said upon learning of my acceptance into graduate school, "I am truly envious". Little did I know how accurate this prosperous scientist was. Although times of academic despair, frustrating research results and personal lows have existed in the past six years, they have also contained the most rewarding times I've yet known.

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ABSTRACT

CHELATION, TRANSPORT AND PHYSIOLOGICAL ROLES OF IRON IN AQUASPIRILLUM MAGNETOTACTICUM.

by

Lawrence Charles Paoletti

University of New Hampshire, May, 1988

Iron is a required nutrient for all living organisms. The magnetic spirillum, Aquaspirillum magnetotacticum possesses the ability to transform this metal to intracellular magnetite. Presented in this dissertation are studies relating to iron chelation and transport mechanisms used by this Gram negative bacterium. In addition, physiological roles of iron, specifically in respiration and the cellular localization of iron-containing respiratory components are also addressed.

INTRODUCTION

Part One

Iron in Biological Systems

This is a "seven part thesis" consisting of chapters defined as units of research. This literature review addresses aspects of iron metabolism not discussed in other portions of this dissertation. It is intended to provide background and related information while avoiding redundancy.

Throughout the living world, iron has an integral role in diverse biological processes. Organisms possess many proteins containing iron. A classification of iron-containing proteins, based on structure and function, has recently been published (8). For a comprehensive list see reference 23. Iron proteins are vital to such widely different biological processes as DNA synthesis, respiration, and in eukaryotes, cell-mediated immunity. The ability of this metal to become easily reduced or oxidized affords it a central position in reactions involving a single electron transfer. The more insoluble Fe(III) or ferric form can be reduced to Fe(II) at a standard reduction-oxidation potential (+0.77 volts) which is close to that of the O_2/H_2O couple (+0.81 volts). Although abundant on Earth, free Fe(III) is not readily available to many microorganisms because it is highly insoluble (K_{sp} for $Fe(OH)_3 = 10^{-38}$ M) at neutral pH values in aerobic environments. In this environment, the available Fe(III) concentration is

approximately 10^{-18}M.

Lactoferrin is an iron-containing glycoprotein found within neutrophils of mammals. This protein plays an important role in mammalian host defense. During an infection, iron released from neutrophils may combine with hydrogen peroxide to yield hydroxyl radicals. The products of this "Haber-Weiss reaction", are bacteriocidal (3). Lactoferrin can also create a bacteriostatic condition by tightly binding ($K_f=10^{24}$ M) available iron (15). Some bacteria, including Bordetella pertussis, Neisseria meningitidis and the protozoan, Trichomonas vaginalis are able to remove and use lactoferrin iron, however (41).

Ferritin and phosvitin are iron containing proteins. The former is found in mammals, plants, fungi and bacteria (44) whereas the latter has been identified in egg yolks of birds, fish, reptiles and amphibians (8). In mammals, ferritins serve to store and maintain the intracellular level of iron necessary for the proliferation of cells such as erythrocytes (2). Ferric iron, located in the mammalian ferritin, is thought to undergo reduction by an electron exchange with ferrous iron, which has entered the protein's inner core (22). Heme-containing ferritins (bacterioferritin) have also been detected in prokaryotes. Cells of Escherichia coli, Azotobacter chroococcum and A. vinelandii contain a bacterioferritin-cytochrome b (or b_1) protein also thought to function as iron storage sites (15, 44). In the latter organism, this protein is thought to be necessary for maintaining high internal iron levels necessary for enzymes involved with nitrogen fixation (44).

The studies of Waring in 1927 (34) and of Waring and Werkman (48)

on the effects of iron limitation on cytochrome content were the first reports concerning iron requirements by prokaryotes. We now know that most microorganisms require between 0.4 to 4.0 μM iron to support basic metabolism (20). Iron usually comprises 0.01 % of the bacterial cell dry weight and is considered a trace nutrient (40). Only the lactobacilli, appear to lack an iron requirement (34).

Because iron is a virulence factor in many infectious diseases (20), considerable research efforts have focused on mechanisms of iron transport by bacterial pathogens. Of course, due to the ease of manipulating enteric bacteria and, consequently, the massive genetic information base underlying this group, many pioneering studies of iron transport have been worked out using strains of Escherichia and Salmonella.

Part Two

Bacterial Iron Transport

Primary transport mechanisms in bacteria include passive diffusion, facilitated diffusion, active transport and group translocation (26). Passive diffusion is a non-specific, energy-independent process which allows entry of small compounds including water, dinitrogen and oxygen (45). At equilibrium, it produces an equal concentration of substrate inside and outside the cell. Facilitated diffusion is similar, however substrate enters via specific, membrane-associated carrier proteins. Due to the involvment of these proteins, this type of transport displays typical saturation kinetics. Many vitamins are internalized via facilitated diffusion (11). Active transport is an energy-requiring process producing greater internal than external substrate concentrations. Substrate-

specific, membrane-bound permeases and binding proteins (located in the cell periplasm) are required for this process. The highly intricate group translocation process involves carrier proteins and enzymes. This energy requiring, concentration gradient-forming system is the mode of transport for many sugars, purines and pyrimidines (45).

Microorganisms have evolved both low and high affinity transport mechanisms for iron. The low affinity iron uptake system is not well studied, but appears to involve simple diffusion of ferric iron through large (ca. 80,000 daltons) proteins and is thought to function at all iron concentrations normally encountered by cells (34). It is conceivable that these proteins may be porins. Porins are large, water-filled channels which permit uptake of small (ca. 600 daltons) molecules (24). The role, if any, of porins in low affinity iron transport awaits experimentation.

Conversely, high affinity iron transport is well characterized and consists of siderophores with their cognate outer membrane receptor proteins (34). Siderophores are low molecular weight (500-1000 daltons), highly ferric iron-specific ligands produced by bacteria and other microorganisms (1, 25, 32, 34). These are released into the environment by cells where they tightly bind ferric iron, and make it available for uptake. Bacterial iron uptake occurs via an energy-dependent, concentration gradient-forming, active transport system (4, 7, 16, 32, 43, 46, 47). Siderophores are usually classified as either phenolates or hydroxamates, although not all iron chelators produced by bacteria fall into these classes (17). A siderophore-like compound is also produced by SV 40-transformed BALB/3T3 cells (19).

For most bacteria, high affinity iron uptake is repressed at iron

concentrations greater than about 1 μM and derepressed during iron starvation. Cells of E. coli, and the endosymbiotic, nitrogen fixers, Erwinia carotovora and Rhizobia leguminosarum displayed energy-driven specific transport of ferrous iron in uptake assays performed anaerobically (7). Lodge and Emery (30) showed ferrisiderophore transport by E. coli cells cultured anaerobically. Are bacteria located in anaerobic habitats (e.g. legume nodules, gastrointestinal tracts or anoxic sediments) capable of specifically transporting ferrous iron or do they prefer chelated ferric iron? Perhaps both iron forms can be used or preference may be dictated by environmental factors as temperature, oxygen tension, pH or Eh'.

The iron transport mechanisms of E. coli are by far, the most throughly understood of all microbial systems. These cells are able to transport iron chelated to a variety of chelators. The six known ligand:receptor protein pairs for this organism are: ferrichrome:Fhu A; coprogen,rhodotorulate:Fhu E; aerobactin:Iut A; citrate:Fec A and enterobactin:Fep A (6). Some E. coli virulence plasmids (pColV-K30, pRJ100 and pColV-K311) carry genes for synthesis of aerobactin and it's receptor protein (6). Recently, the fur (ferric uptake regulation) gene product has been shown to negatively regulate siderophore producing gene systems including the aerobactin-mediated transport system contained on pColV-K30. This repressor requires ferrous iron (or Mn(II), Zn(II), Co(II), Cd(II)), and will not bind to "iron" operators without one of these divalent cations (6).

The cellular site and mechanism of iron reduction is unclear (6). Three possible mechanisms of iron release have been advanced. One involves dissociation of the ferrisiderophore complex at the cell

surface, leaving the free ligand external to the cell (13, 31). A second mechanism invokes the hydrolysis of the internalized ligand by ferrisiderophore esterases (12). In E. coli cells, the esterase is the product of the fes gene. The cellular location of this enzyme is not presently known. A third proposed mechanism of iron release involves ferrisiderophore reductases (4, 14, 29, 33). These reduce ferric iron and, due to the lowered affinity of siderophores for ferrous iron (e.g. log K_f for hydroxamic acids is 8 and 28 for Fe(II) and Fe(III), respectively; 7), release is assured. In some cases, the ligand is unaltered and is again released from the cell to repeat the sequestering cycle (15).

Although questions remain, research on high affinity iron transport (especially in E. coli) has now progressed toward a working knowledge at the molecular and genetic level.

"The amazing versatility of bacterial and fungal metabolism suggests that additional novel iron harvesting mechanisms remain to be discovered in the microbial world."

-J. B. Neilands (35)

It is commonly known that the high affinity iron transport mechanisms of enteric bacteria are induced (or derepressed) under conditions of iron depletion; 1 μM or less is commonly cited as the critical value (34). This is not a universal pattern among bacteria, however. Schizokinen, a hydroxamate siderophore produced by cells of Bacillus megaterium was first identified as a by-product of cell division (28). Although schizokinen yields were shown to increase as culture iron levels were depleted (10), the initial experiments utilized

media which contained 36 μM iron (28). This was the first report of an iron chelator produced by cells cultured with iron levels greater than 1 μM iron. Cells of Azotobacter chroococcum strain 184 optimally produced hydroxamates when cultured with 0.75 μM iron (36). Moreover, these cells did not produce hydroxamates when iron was completely omitted from the growth medium. Stemphyloxins, a class of phytotoxic iron chelators produced by the fungus Stemphylium botryosum, are optimally produced at iron concentration of 2 mg/l and are repressed at both low (0-1 mg/l) or high (>8 mg/l) iron (32) The pathogen, Haemophilus influenzae produced hydroxamate(s) optimally when cultured with 100 μM ferric nitrate (39). Therefore, the tenet that siderophores are produced solely during iron limitation is not applicable to all microorganisms especially those inhabiting unusual environments and/or possessing unique metabolic traits.

Part Three

Magnetotactic Bacteria and Iron

The prototypic magnetotactic bacterium, Aquaspirillum magnetotacticum strain MS-1 is able to convert extracellular iron into magnetite (9). Whether these cells are able to produce iron chelating compounds was not known. Results contained in chapter one of this thesis, **Hydroxamate production by Aquaspirillum magnetotacticum**, indicate that culture fluids from magnetic cells grown with 20 and 40 μM but not with 5 μM iron contain an iron chelator (37). Interestingly, mutant strain NM-1A cells produced hydroxamate at both low (5 μM) and high (20 μM) iron. The effects of culture iron on expression of outer membrane proteins in these strains are also

presented.

Radioactive iron has been used in studies of microbial iron assimilation (7, 13, 16, 30, 42, 46, 47). In chapter two, **Initial characterization of the iron uptake system of Aquaspirillum magnetotacticum**, data are presented which are supportive for an energy-dependent active transport system for iron. Iron uptake displayed temperature dependence and was more specific for ferric than for ferrous iron.

Once iron is internalized, it must be reduced to the ferrous form to be available for metabolic uses and, in the case of magnetic bacteria, for magnetite synthesis (21). Enzymes which reduce uncomplexed or complexed iron have been detected and isolated from many bacteria. Chapter three of this thesis, **Iron reduction by Aquaspirillum magnetotacticum**, addresses enzymatic iron reduction by cell free extracts. Most (77 %) of the total iron reductase activity was located in the cell periplasm of strain MS-1. Soluble reductases were able to reduce iron uncomplexed or complexed to a variety of ligands and the activity of membrane-associated reductases was unaffected by respiratory inhibitors. Due to their abundancy in the periplasm, these enzymes may play an important role in facilitating iron transport in this organism.

In the course of work in this thesis, it was determined that **Freeze-thawing Aquaspirillum magnetotacticum cells selectively releases periplasmic proteins** (38). This method was used in studies (e.g. Chapters 3, 5, 6) of periplasmic proteins and those requiring separation of proteins in different cellular compartments.

Although most of the internalized iron is incorporated into

magnetosomes, some is directed towards synthesis of components of respiration. For example, Chapter five of this thesis, **Periplasmic c-type hemoproteins of Aquaspirillum magnetotacticum**, describes soluble hemoproteins detected in this organism. Cytochromes of the c-, and cd_1-types were detected in soluble fractions of denitrifying strain MS-1 cells.

A 17,000 dalton protein was found to be common to all cellular fractions of this organism including membranes, cytoplasm and periplasm. This protein, isolated from periplasm by SDS-PAGE, exhibited a difference spectrum typical of c-type hemes. To investigate possible relationships between membrane-associated proteins of similar mass and this soluble hemoprotein, immunological studies were undertaken. Chapter six, **Antigenic relatedness of a periplasmic c-type cytochrome and an outer membrane protein in Aquaspirillum magnetotacticum**, contains evidence for antigenic relationships between these proteins.

Batch cultured cells undergo changes in their metabolism as culture conditions vary during growth. Cells grown in continuous culture are desirable for metabolic studies (5, 18, 27). This culturing method is designed for precise control of parameters including nutrients, pH, and temperature and is also used to regulate cell growth rates. This culturing method provided, for other studies such as iron uptake, a continuous supply of viable cells held at constant physiological state. Of somewhat incidental interest to the objectives of this thesis but potentially of significant value, Chapter seven, (**Iron relieves nitrate limitation in continuous cultures of Aquaspirillum magnetotacticum**) contains evidence that between 8 and 16

μM iron, cells shift from predominately nitrate-respiring to an oxygen-utilizing mode of respiration: a shift triggered by available iron concentration.

Lastly, attempts to purify the hydroxamate chelator produced by A. magnetotacticum are presented in Appendix A, **Purification of hyroxamate from culture fluids of Aquaspirillum magnetotacticum**. Although conventional methods for the isolation of hydroxamate proved unsuccessful, further attention to this project is warranted.

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CHAPTER ONE

CHAPTER ONE

Hydroxamate Production by Aquaspirillum magnetotacticum.

Abstract

Spent culture fluids from Aquaspirillum magnetotacticum strain MS-1 grown at high (20 μM) but not low (5 μM) iron contained material giving a positive hydroxamate test. Six major outer membrane preoteins were detected by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An addition 55,000 dalton iron-repressible outer membrane was present in strain MS-1 cultured at low but not high iron. The presence of this membrane protein did not correlate with hydroxamate production over the range of iron concentrations tested. Culture fluids from strain MS-1 which were hydroxamate positive augmented the growth of a Salmonella typhimurium siderophore-deficient (enb-7b) mutant in low iron medium.

Introduction

Numerous bacterial proteins including cytochromes, catalases, peroxidases, superoxide dismutases, ribotide reductases and nitrogenases contain iron (15). Due to its insolubility at neutral pH under aerobic conditions, iron is usually unavailable for direct uptake by cells (16). Under conditions of low iron (less than 1 μM), many microorganisms produce iron chelators, termed siderophores (16). These are assimilated into gram negative cells by means of specific receptor

proteins located in the outer membrane (16, 18, 20).

Siderophores have been detected in spent culture fluids of aerobes and facultative anaerobes, but are apparently not produced by strict anaerobes or the lactic acid bacteria (15, 16). No information exists concerning siderophore production by obligate microaerophiles.

Aquaspirillum magnetotacticum (13) is a gram negative obligately microaerophilic chemoheterotroph which contains 2.0 % of its dry weight as iron. Although this organism contains iron in proteins and hemoproteins, most of this metal is compartmentalized within its magnetosomes, which are intra-cellular enveloped crystals of the iron oxide, magnetite (3). Virtually nothing is known of the manner in which cells of this organism sequester iron. However, both in its natural habitat and its culture medium the total iron concentration is 20 uM. In nature, the iron may be complexed with humic substances or plant derived organic acids. In the culture medium used, iron is chelated with quinic acid.

This study was initiated to determine whether A. magnetotacticum utilizes a high affinity (siderophore) system similar to those employed by other gram negative organisms for iron aquisition.

Materials and Methods

Bacterial strains and growth conditions. Cells of A. magnetotacticum strains MS-1 (ATCC 31632) and NM-1A were cultured microerobically in chemically defined growth medium (MSGM) as previously described (2). The iron source was ferric quinate provided at concentrations of 0, 5, 10, 20 or 40 μM. $FeSO_4$ was omitted from the culture medium mineral

solution, and for studies involving spectrophotometric analysis of supernatant fluids, resazurin was omitted. Ferric chloride-sodium citrate mixture with citrate and iron in a molar ratio of 1:1 or 20:1 (iron concentrations of either 5 or 20 μM, as indicated) was used in lieu of ferric quinate. Without added iron, MSGM contained 0.35 μM iron as determined by the ferrozine method (18). No attempts were made to completely deferrate the culture medium.

Salmonella typhimurium LT-2 enb-7b, an enterobactin deficient mutant (a gift from J.B. Neilands, University of California at Berkeley) and S. typhimurium ATCC 14028 were maintained on nutrient agar slants and subcultured bimonthly. To promote siderophore production, S. typhimurium 14028 was cultured for 48 h at 37°C on a rotary shaker in 0.25% (w/v) Casamino Acids (Difco) solution containing 0.2 mM $MgCl_2$, and adjusted to pH 7.5 (18).

Isolation of outer membrane proteins. A. magnetotacticum strains MS-1 and NM-1A were grown to early stationary phase in 1 liter batch cultures. Cells were harvested by centrifugation (7,000 x g, for 15 min at 4°C), and resuspended in 10 ml of 50 mM potassium phosphate buffer (pH 6.8). Outer membrane proteins were isolated by the procedure of Schnaitman (17). Briefly, DNase and RNase (Sigma) were each added to cell suspensions at a final concentration of 0.01 % (wt/vol). Cells were disrupted by two passes through a pre-cooled French pressure cell (16,000 lbs/in^2). Unbroken cells and cellular debris were removed by centrifugation at 7,000 x g for 15 min at 4°C. The resulting supernatant fluid was ultracentrifuged at 200,000 x g for 60 min at 4°C. The brown pellet, containing both inner and outer cell

membranes, was resuspended in 10 ml of 10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES), pH 7.4, containing 2 % (v/v) Triton X-100 and 10 mM $MgCl_2$. The unsolublized outer membrane fraction was collected by ultracentrifugation (200,000 x g, 60 min at 4°C), and washed once in 10 mM HEPES (pH 7.4) to remove residual Triton X-100. The solubilized cytoplasmic membrane proteins were precipitated with cold 95 % ethanol overnight at -12°C, and collected by centrifugation (7,000 x g, 30 min at 4°C). Protein determinations were made with the procedure of Lowry et al. (11) using bovine serum albumin as a standard. The activity of succinic dehydrogenase (SDH), a cytoplasmic membrane enzyme, was assayed in each cell fraction (5) to assess the purity of the outer membrane fraction.

Electrophoresis and analysis of OMPs. Outer membrane proteins and molecular weight standards (Bio-Rad) were solubilized and separated by the electrophoretic methods of Laemmli (9). Proteins were stacked in a 4 % acrylamide gel at a constant current of 10 mA. The current was increased to 20 mA as proteins entered a 12 % acrylamide separating gel. Proteins were stained with Coomassie brillant blue and quantitated using a Helena Quick Scan R&D densitometer (Helena Laboratories, Beaumont, Texas) at 590 nm.

Detection of iron chelators. Spirilla were grown in 500 ml batch cultures containing iron as ferric quinate at 5, 20 and 40 μM or as ferric citrate with the molar ratio of citrate to iron either at 20:1 or 1:1 (20 μM Fe). Post-growth culture fluids were freed of cells by centrifugation (7,000 x g, 15 min) and vacuum filtered using 0.45 μm Metricel GA-6 filters (Gelman Sciences, Michigan). The fluids were

concentrated to 1/45 their original volume by flash evaporation at 35°C. Each sample was then carefully adjusted to pH 7.0 with 1 N HCl or NaOH, filter sterilized and stored at 4°C until assayed. Uninoculated culture media at each iron concentrations and postgrowth culture fluids (concentrated 1/10 original volume) from S. typhimurium 14028 were identically prepared as controls.

The Arnow test (1) was employed for the detection of phenolate type iron chelators. The positive control were 400 μM catechol (Sigma) and 1.5 mM 2,3-dihydroxybenzoic acid (Aldrich); the negative controls were 1.5 mM solutions of acetohydroxamic acid (Aldrich) and deferoxamine (a generous gift from Ciba-Geigy, New Jersey). A modification of the Csaky reaction was used to detect secondary hydroxamic acids (7). The negative controls were catechol and DHB; the positive controls were acetohydroxamic acid and deferoxamine.

Siderophore activity. The effect of spirillum culture fluids on growth of the enterobactin-deficient S. typhimurium strain enb-7b was examined. To each sidearm flask containing 100 ml of Davis minimal medium (4) containing no added iron was added 1.0 ml of an overnight culture of the S. typhimurium enb-7b strain grown in Davis minimal medium without iron or citrate. To each inoculated test flask, 1 ml of either pre- or postgrowth fluids from strain MS-1 cultured in MSGM containing 5 or 20 μM ferric quinate was added. To each control flask, 1.0 ml of either S. typhimurium 14028 culture supernatant fluid, or Davis minimal medium was added. Growth of the S. typhimurium enb-7b mutant at 37°C (shaking water bath) was monitored at $O.D._{660}$.

Results

Outer membrane proteins and iron. Six major OMPs ranging from 16,400 to 64,500 daltons were produced by strains MS-1 and NM-1A (Fig. 1). The OMP preparation appeared to be relatively free of cytoplasmic membrane proteins, in that it contained only 7.0 % of the total SDH activity of the various cell fractions. When cultured at low (0 or 5 μM) but not at high (10, 20 or 40 μM) added ferric quinate, strain MS-1 produced a 55,000 dalton OMP (Fig. 1, lanes 4-8). This 55,000 dalton iron-repressible OMP (IROMP) comprised 13.0 and 4.0 % of the total major OMP of cells cultured at 0 and 5 μM added ferric quinate, respectively. This IROMP also comprised 1.0 % of the total OMP of strain NM-1A cells grown with no added ferric quinate (Fig. 1, lane 1). Three minor OMPs (72,000, 76,000, and 85,000) not present at low iron, were produced by cells of each strain cultured with 20 or 40 μM ferric quinate (Fig. 1, lanes 2, 3, 7, 8).

Strain MS-1 cells cultured in medium with 400 μM sodium citrate and 20 μM ferric chloride produced in addition to the 55,000 ddalton IROMP, another of 58,000 dalton (Fig. 2, lane 1). Cells cultured with 20 μM sodium citrate and 20 μM ferric chloride produced neither the 58,000 nor the 55,000 dalton IROMP (Fig. 2, lane 4). Cells grown with either 5 μM ferric quinate or 20 μM ferric citrate with the citrate-to-iron molar ratio of 20:1 (e.g. conditions of low iron availability) produced the 55,000 dalton IROMP (Fig. 2, lanes 1 and 2). This protein was absent from cells cultured with 10 μM or more ferric quinate (Fig. 1, lanes 6-8) or at ferric citrate concentrations of 20 μM with the citrate-to-iron molar ratio of 1:1 (Fig. 2 lanes 3 and 4).

Siderophores. Catechol-type iron chelators were not detected in spent culture fluids of strains MS-1 or NM-1A (by means of the Arnow reaction). Hydroxamate-type iron chelators were produced by cells of each strain (Table 1) as evidenced by positive Csaky tests. Hydroxamates were detected in culture media of cells grown at 20 or 40 μM ferric quinate. Surprisingly, spent medium from strain MS-1 cultured with less (5 μM) ferric quinate consistantly failed to give a positive Csaky reaction (Table 1). Culture fluids from cells of strain NM-1A grown at either 5 or 20 μM ferric quinate were positive (Table 1), however.

Culture fluids from strain MS-1 cultured with 20 μM ferric citrate at a citrate-to-iron molar ratio of 1:1 were positive in the Csaky test whereas those obtained from cells grown at a 20:1 molar ratio were not (Table 1).

Siderophore activity. The enterobactin-deficient S. typhimurium enb-7b mutant did not grow in low iron medium in the absence of exogenously supplied chelators (Fig. 3). Phenolate or hydroxamate (12, 15) siderophores have been shown to allow for the growth of this mutant in low iron medium. Culture fluids from S. typhimurium 14028 (wild type) markedly stimulated the growth of the S. typhimurium enb-7b mutant whereas uninoculated sterile Davis Minimal Medium (negative control) treated similarly had little effect (Fig. 3). Growth obtained using pre- or postgrowth fluids from spirilla cultured with 5 μM ferric quinate or those of spirillim pregrowth culture fluids containing 20 μM ferric quinate did not exceed that obtained using uninoculated Davis Minimal Medium (Fig. 3). At 10 h, the enb-7b mutant supplied with

postgrowth supernatant fluid from spirillum strain MS-1 cells cultured with 20 μM ferric quinate showed a 50 % higher culture absorbance than when supplied with Davis Minimal Medium (negative control) or 20 μM ferric quinate pregrowth fluids (Fig. 3).

Discussion

Our results indicate that magnetic cells of A. magnetotacticum produced hydroxamate material when cultured at 20 or 40 μM added ferric quinate but not when grown with 5 μM ferric quinate. The non-magnetic mutant, strain NM-1A produced hydroxamates at both iron concentrations tested (5 and 20 μM ferric quinate). Because these results were unexpected in the light of iron concentration effects on siderophore production by enteric bacteria (10, 12, 15, 16), we repeated this study using an alternate source of iron, ferric citrate. At physiological pH and with citrate in a 20-fold molar excess, the ferric citrate complex can be expected to exist in a highly polymerized state (14, 16). A. magnetotacticum strain MS-1 cells responded in their hydroxamate production to the available iron concentration in their culture medium. At 20 μM iron supplied as ferric citrate, cells produced hydroxamates when the molar ratio of citrate to iron was 1:1 but not 20:1. Since the latter situation represents low available iron for A. magnetotacticum, these results corroborated those obtained with ferric quinate; magnetic cells of this species produce secondary hydroxamates only when cultured under iron sufficient conditions.

Although in enteric bacteria, siderophore synthesis is derepressed by iron deficiency, hydroxamate synthesis by cells of A.

magnetotacticum is apparently repressed by iron deficiency or is induced by available iron.

With low available iron, many bacteria synthesize OMPs which function as receptors for siderophores (8, 10, 15, 16, 18). The 55,000 dalton IROMP in A. magnetotacticum may not function in iron transport via hydroxamates as its synthesis was repressed at iron concentrations necessary for hydroxamate production. Thus, it may be a component of another iron uptake system not involving hydroxamates. Cells of strain NM-1A did not produce the IROMP but did produce hydroxamates, suggesting that mutation(s) resulting in the loss of magnetite production may be associated with the gene(s) directing the synthesis of the IROMP.

In the enteric bacteria, iron storage proteins or those involved with nonspecific iron transport are usually large (8, 16). The 72,000, 76,000 and 85,000 dalton OMPs detected in cells of A. magnetotacticum cultured at 20 or 40 μM ferric quinate may serve a role in iron metabolism comparable to those OMPs of similar size and produced under similar conditions by the enterics (16). Alternatively, their coordinate production under conditions in which cells also produce the hydroxamate suggests these may be involved in hydroxamate secretion and/or binding.

The 58,000 dalton IROMP produced by cells of A. magnetotacticum cultured with citrate may be a component of a citrate-mediated iron uptake system similar to that of Mycobacterium smegmatis (14) or E. coli (16, 20).

The fact that only culture fluids which tested positively for

hydroxamates stimulated growth of the S. typhimurium enb-7b mutant is consistent with a physiological role of this material in iron transport (e.g. as a siderophore) in A. magnetotacticum.

Two of the three types of Azotobacter vinelandii siderophores are produced to some extent by cells cultured with 25 μM iron (6).

We do not presently know why hydroxamate is produced at high iron and less so at low iron by A. magnetotacticum strain MS-1. Recently we have detected hydroxamate production at high (20 μM) iron by Aquaspirillum bengal, A. serpens, and A. polymorphum (L. Paoletti and R. Blakemore, unpublished results), which are not magnetic. Thus, the magnetic spirillum appears not to be unique in this respect. This is an unusal pattern and although not many published studies include results of hydroxamate analysis at both high and low iron, this appears to be the first report of bacterial hydroxamate production at high iron.

Siderophore production at high iron may be common among free-living organisms which accumulate this metal or require it for metabolism.

Acknowledgements

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Table 1. Hydroxamate and IROMP production by *A. magnetotacticum*.

Strain and supplement (μM)	Hydroxamate production (μM)[a]	Production of 55,000 dalton IROMP[b]
Strain MS-1, ferric quinate		
5	<5	+
20	49	-
40	53	-
citrate:iron[c]		
20 (1:1)	36	-
20 (20:1)	<5	+
Strain NM-1A, ferric quinate		
5	47	-[d]
20	77	-

[a]Values are deferoxamine equivalents.

[b]+, production; -, no production.

[c]Values in parentheses indicate the ratio of citrate to iron.

[d]Inferred result based upon absence of the IROMP from electrophoretic protein profiles of strain NM-1A cells cultured with 0, 20, and 40 μM added ferric quinate.

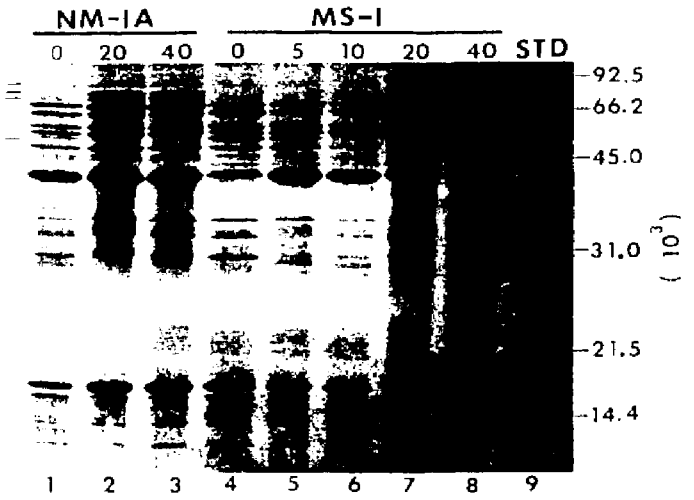

FIG. 1. SDS-PAGE of OMP profiles from A. magnetotacticum strains MS-1 and NM-1A cultured at various iron concentrations. Each lane contained 30 μg of purified (17) OMP. Dashes indicate the positions of the 55,000 IROMP, 72,000, 76,000 and 85,000 dalton proteins. Lane 9, on the right, contained the molecular mass standards (in kilodaltons) indicated.

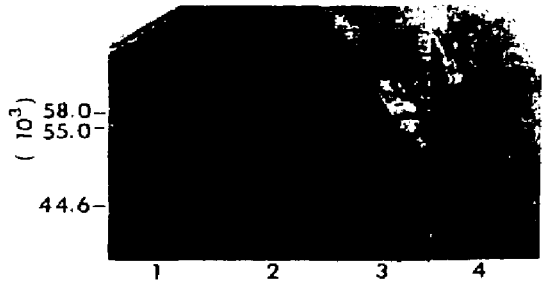

FIG. 2. SDS-PAGE of OMP profiles from A. magnetotacticum strain MS-1 cultured with citrate to iron at molar ratios of 20:1 and 1:1, lanes 1 and 4, respectively; or with 5 or 20 μM ferric quinate, lanes 2 and 4, respectively. Each lane contained 30 μg of purified (17) OMP. The positions of the iron citrate induced OMP (58,000 dalton), the IROMP (55,000 dalton) and the major OMP (44,600 dalton) are indicated.

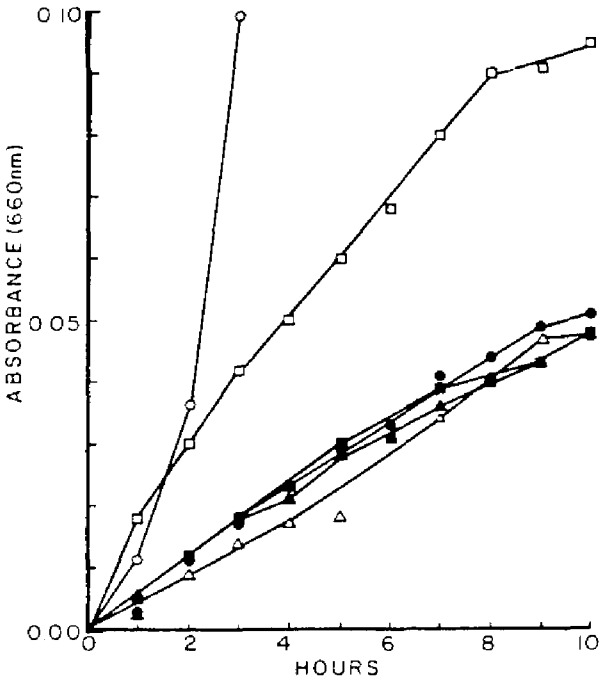

FIG. 3. Growth response of S. typhimurium LT-2 enb-7b mutant cultured in Davis minimal medium supplemented with pregrowth culture medium containing 20 μM ferric quinate, ■ ; 5 μM ferric quinate, ▲ ; or postgrowth culture fluids from strain MS-1 grown with 20 μM ferric quinate, □ ; 5 μM ferric quinate, △ . Controls contained Davis minimal medium (negative control), ● ; or postgrowth fluids from S. typhimurium 14028 (positive control), ○ .

CHAPTER TWO

CHAPTER TWO

Initial Characterization of the Iron Uptake System of *Aquaspirillum magnetotacticum*

Abstract

Iron-starved *Aquaspirillum magnetotacticum* strain MS-1 cells transported ferric iron under aerobic assay conditions at 30°C, with apparent Km and Vmax values of 35 and 1.25 nmol/min/mg biomass, respectively. Active transport is apparently used by these cells to transport iron as this process was inhibited by metabolic inhibitors 2,4-dinitrophenol, sodium azide and mercuric chloride and was temperature-dependent.

Introduction

Aquaspirillum magnetotacticum strain MS-1 cells cultured in a chemically defined medium containing chelated iron (1), contain 2 % of their dry weight as iron (4). Iron is predominately in the form of magnetite, the production of which is dependent upon available oxygen (2) and iron concentrations. These cells use ferric iron in substrate dissimilation (10). They also produce hydroxamate-type chelators at high (20 or 40 μM) iron (8). These findings underscore the importance of iron to this organism. However, at the time this work was begun, little was known concerning the mechanisms of iron transport by these bacteria.

Materials and Methods

Radioactive iron uptake. Magnetotactic cells collected from the outflow of a continuous culture vessel ($D=0.075\ h^{-1}$ and $t_D=9.2$ h), were

harvested by centrifugation (13,000 x g, 15 min, 22°C) and washed once with 200 ml uptake buffer. Uptake buffer was prepared by adding 100 g wet wt of Chelex 100 resin (BioRad, Richmond, CA) to 1 l of concentrated (10-fold) magnetic spirillum growth medium (1). This solution was allowed to mix overnight at 4°C. Resin was removed by filtration (Whatman #1 filter paper followed by 0.45 μm membrane filters), the medium was adjusted to pH 6.75 and autoclaved. Each day, this was diluted 1 part to 9 parts with Milli-Q H_2O and used as the uptake buffer. Washed cells were suspended in 180 ml uptake buffer ($O.D._{660\ nm}$ ca. 0.6) and 25 ml of cells were placed in each of several acid washed 125 ml Erlenmeyer flasks. Prior to the addition of ^{59}Fe, cells in the flasks were allowed to equilibrate for 1 h in a 30°C water bath (Precision Scientific, Chicago, IL) with gentle mixing (90 r.p.m.). Cells were provided with radioactive iron (Dupont/New England Nuclear, North Billerica, MA) at a final concentration of 20 μM (sp. act.=0.04 μCi/μg) as ferric chloride. At timed intervals, 1 ml of cells was removed and filtered through a 0.2 um polycarbonate membrane filter (Nuclepore). Filters were rinsed 5 times with 2 ml of 50 mM EDTA then removed and dried under a heat lamp. Dried filters were counted for 1 min in a Beckman model 5500 gamma counter. The filtration apparatus was rinsed with 100 mM sodium citrate between samples. Cell biomass was estimated by collecting washed cells onto a preweighed filter, drying the filter in a 60°C oven for 24 h and weighing. Results are the means of duplicate samples representative of at least two experiments.

Results

Iron uptake by *A. magnetotacticum* strain MS-1. Magnetotactic cells washed and suspended in Chelex-treated buffer (e.g. held under iron limitation) for 1 h sequestered iron (uncomplexed or chelated 1:1 with quinic acid) at a maximum rate of 0.63 nmol/min/mg biomass for 15 min, after which the rate progressively decreased (Fig. 4). Uptake by cells of iron supplied as ferric quinate (FeQ) slowed abruptly at 30 min whereas with unchelated iron the rate slowed more progressively over a 1 h interval (Fig. 4). Maximal iron uptake occurred between 1.5 to 3.0 h with less than 2 % of the total iron uptake occuring after 1.5 h. Of the total iron supplied, 12-27 % was taken up by cells at 1 h.

Effects of oxygen, temperature on iron uptake. Concentrated magnetotactic cells remained motile in buffer for at least 24 h. Therefore, iron uptake assays were performed under aerobic conditions. However, when harvested and assayed at lowered values of O_2 (e.g. with the assay sparged with nitrogen gas), cells accumulated iron at 47-84 % greater amounts (Fig. 5).

The optimal temperature range for iron uptake during the first 15 min of exposure to iron was 20 to 30°C with an optimum initial rate at 25°C (Fig. 6). Cells incubated at 45°C became non-motile and did not take up iron.

A. magnetotacticum strain MS-1 cells transported ferric iron inward to 153 % higher final concentrations and at 133 % higher rates than they did ferrous iron (Fig. 7).

Effects of iron concentrations and metabolic inhibitors on iron uptake. Both initial rates of uptake and final concentrations of iron increased

proportionally with extracellular iron concentration (Fig. 8). Samples from control flasks containing 20 μM $^{59}FeCl_3$ without cells did not contain non-filterable iron particles. However, with 40 μM $FeCl_3$ added to the uptake medium (no cells) nonspecific binding, due presumably to formation of ferric oxyhydroxide precipitates, increased to about 8 nmol Fe/ml by 1.5 h. Counts obtained in experiments with 40 μM iron were corrected for trapped insoluble precipitates. Double-reciprocal of iron uptake rates (presented in Figure 8) as a function of iron concentration yielded a Km for $FeCl_3$ of 35 μM and a Vmax of 1.25 nmol/min/mg biomass (correlation coefficient = 1) for magnetotactic cells (Fig. 9).

Cells in batch culture with 40 uM FeQ incorporated iron in a 3-4 fold greater amounts than did those cultured with 5 or 20 μM FeQ (Fig. 10).

Mercuric chloride, 2,4-dinitrophenol and sodium azide at 0.05, 1.0 and 25 mM respectively, totally inhibited iron uptake by strain MS-1 cells (Table 2). Inhibition of iron uptake was not observed by cells treated with 0.5 mM 2,4-dinitrophenol and only 30 % inhibition resulted from exposure of cells to 1 mM sodium arsenate (Table 2).

Discussion

Inhibition by metabolic poisons and temperature-dependence of iron uptake as noted in this study are characteristic of active transport by microorganisms (3, 7, 9, 11, 12). Corynebacterium diphtheriae cells optimally transport iron within a very narrow temperature range with a maximum initial velocity at 40°C (9). Strain MS-1 cells transported iron at 10-15 centigrade degrees lower and over a wider temperature

range than did C. diphtheriae cells. Interestingly, both C. diphtheria and A. magnetotacticum transported ferric iron to a greater extent than they did ferrous iron (9). This was an unexpected finding as most organisms, including fungi (7), usually take up the more soluble ferrous form more readily.

Bacteria cultured with greater amounts of iron generally display a decrease in iron uptake (11), presumably due to a saturation effect. Iron uptake by the magnetic spirillum was enhanced by cells cultured with higher concentrations of iron. This is consistent with earlier findings (L. Paoletti, M.S. thesis, University of New Hampshire, 1984; N. Blakemore, unpublished results) of a positive correlation between the average number of magnetosomes per cell and culture iron concentrations.

Cells of A. magnetotacticum accumulates copious iron as compared to the enterics. Therefore, we were not surprised that the Km and Vmax values obtained with strain MS-1 cells were significantly greater than those reported for iron uptake via ferric enterobactin in Escherichia coli (3).

Magnetotactic bacteria are well known to compartmentalize iron internally as magnetite. When cultured at high (20 μM) iron, A. magnetotacticum strain MS-1 cells produced a hydroxamate-type chelator which is believed to serve as a siderophore for this organism (8). Ferric iron may well be reduced prior to passage through the cytoplasmic membrane as most (77 %) of the cell's total iron reductases were recovered from the periplasm (L. C. Paoletti and R. P. Blakemore, in press). Using Mössbauer spectroscopy, evidence for two iron forms, a low density hydrous ferric oxide (accomplished by a reoxidation of

previously reduced iron) and a high density hydrous ferric oxide or ferrihydrite (formed by dehydration and reduction), exists in cells as precursors to magnetite (5). Magnetite particles are enveloped by a lipid bilayer membrane containing proteins some of which are unique to it (6).

Under aerobic assay conditions at 30°C, iron-starved magnetotactic cells transported ferric iron with an apparent Vmax of 1.25 nmol/min/mg biomass. Active transport is apparently used by these cells to transport iron as this process was energy dependent, evidenced by inhibition by metabolic poisons and was temperature-dependent. Details of the transport system are not well known, however.

Acknowledgments

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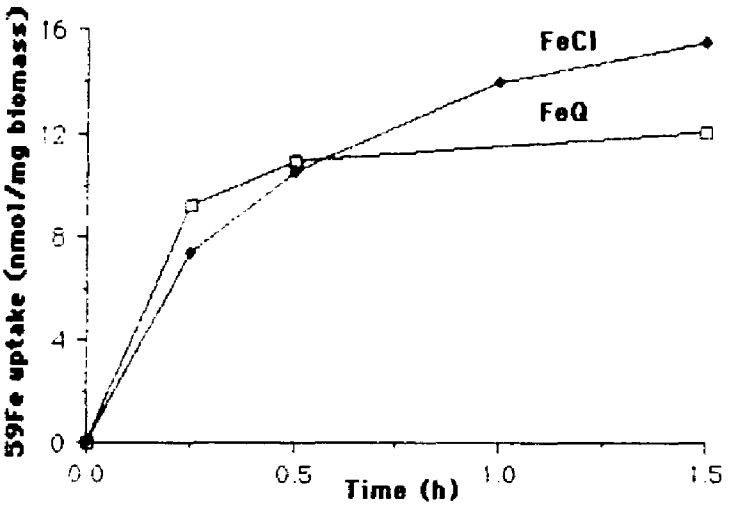

FIG. 4. Uptake of $^{59}FeCl_3$ or ^{59}FeQ by iron starved cells of Aquaspirillum magnetotacticum strain MS-1. Results presented are the means of duplicate samples of at least two experiments.

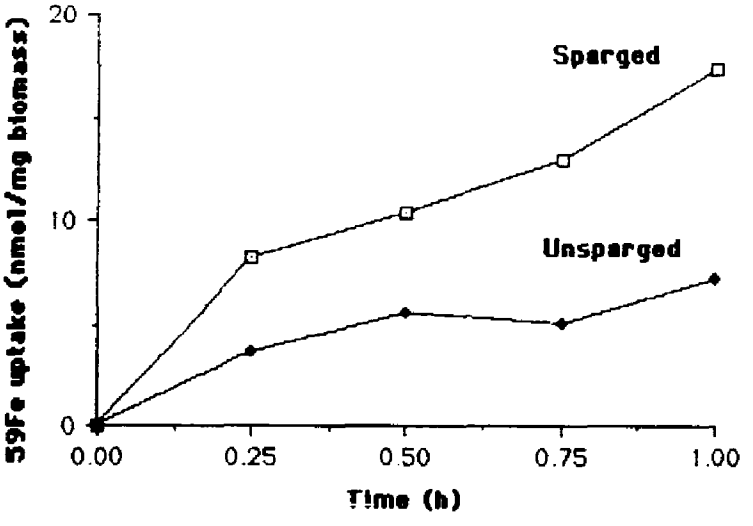

FIG. 5. Uptake of radioiron by sparged and unsparged preparations of Aquaspirillum magnetotacticum strain MS-1.

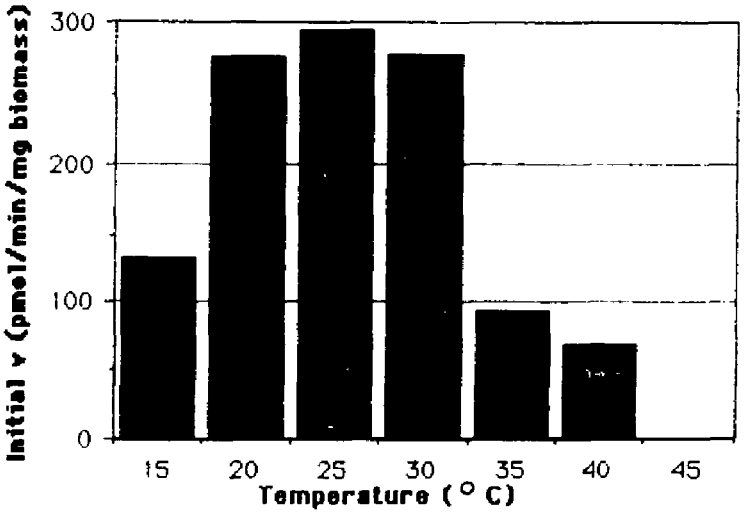

FIG. 6. Effects of temperature on iron uptake by Aquaspirillum magnetotacticum strain MS-1. Cells were acclimated to each temperature for 30 min prior to the beginning of the experiment.

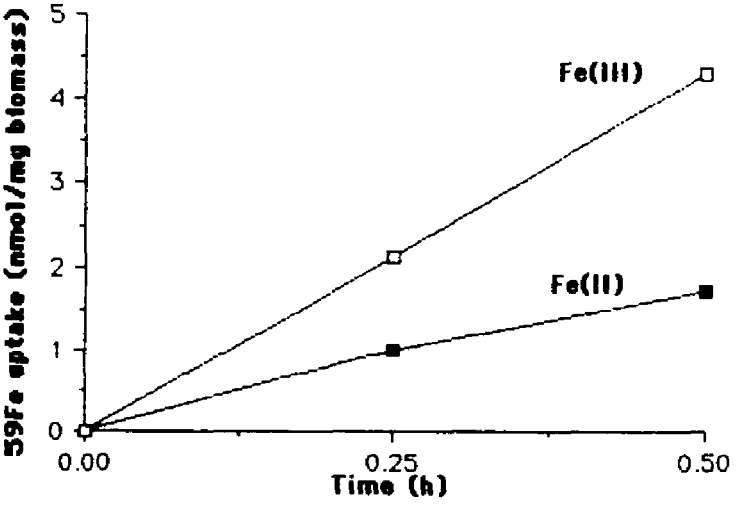

FIG. 7. Specificity of ferric iron by cells of Aquaspirillum magnetotacticum MS-1. Ferrous iron was made by adding 200 μM freshly prepared ascorbic acid to 20 μM radioiron. Final iron concentration in the assay was 20 μM.

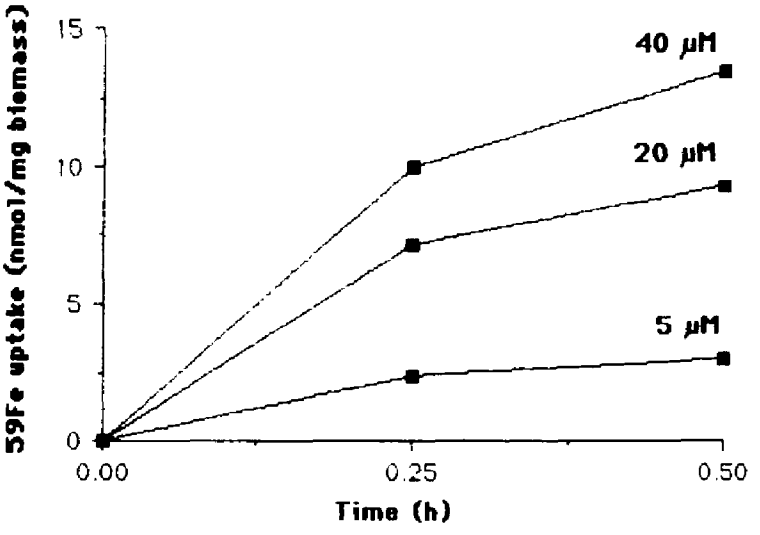

FIG. 8. Uptake by Aquaspirillum magnetotacticum strain MS-1 cells of 5, 20 or 40 µM added $^{59}FeCl$.

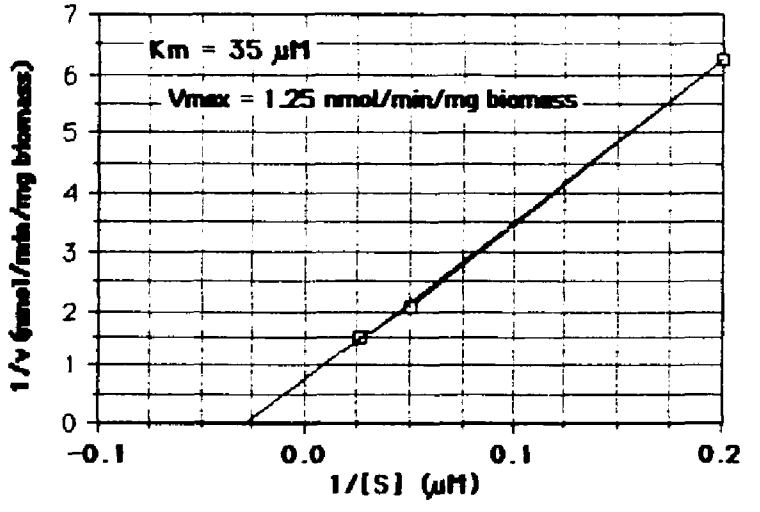

FIG. 9. Lineweaver-Burke plot of initial $^{59}FeCl_3$ uptake rates by Aquaspirillum magnetotacticum strain MS-1 cells (shown in Fig. 8).

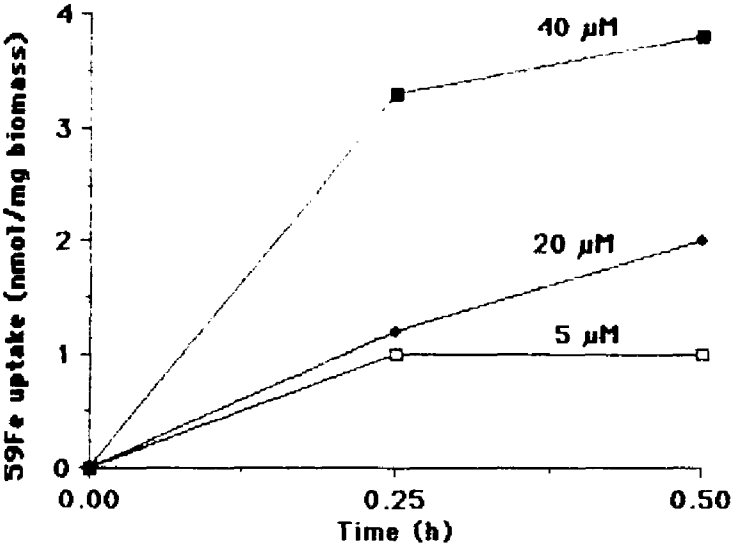

FIG. 10. Iron uptake by Aquaspirillum magnetotacticum strain MS-1 cells batch cultured at 30°C with 5, 20 or 40 µM FeQ.

Table 2. Effects of metabolic inhibitors on iron uptake by Aquaspirillum magnetotacticum strain MS-1.

Inhibitor	Final concn. (mM)	% Inhibition at 30 min[a]
Nil		0
2,4-dinitrophenol	0.5	0
	1.0	100
Sodium azide	25	100
Mercuric chloride	0.05	100
Sodium arsenate	1.0	30

[a]Assays were performed as described in Materials and Methods. Inhibitors were added 30 min prior to the addition of iron.

CHAPTER THREE

Chapter Three

Iron Reduction by Aquaspirillum magnetotacticum.

Abstract

Iron reductase activity in cell extracts of Aquaspirillum magnetotacticum strain MS-1 (wild type) or non-magnetotactic mutant strain NM-1A was located primarily in the periplasm. Cytoplasm contained 20-35 % and membrane fractions 3 % of total iron reductase activity detected. Iron reduction was reversibly inhibited by oxygen, required β-NADH and was enhanced by flavins. Reduced disulfide bonds and uncomplexed sulfhydryl groups were necessary for reductase activity. Respiratory inhibitors did not affect iron reductase activity. Iron complexed with quinic acid, dihydroxybenzoic acid, acetohydroxamic acid, citric acid, or deferrioxamine B was reduced by soluble iron reductases of strain MS-1.

Introduction

Enzymatic reduction of ferric iron to the more soluble ferrous form may be an important and common feature of bacterial iron assimilation (1-4, 7-9, 11, 16). Some bacteria also couple iron reduction to substrate dissimilation (6, 15).

Magnetotactic bacteria assimilate extracellular ferric iron by incompletely understood means (5, 13). They convert much of it to intracellular magnetite (a mixed-valence iron oxide) by steps involving iron reduction (5). One species available for pure culture studies, Aquaspirillum magnetotacticum strain MS-1, is also apparently capable

of dissimilatory iron reduction because it exhibits iron respiration-driven proton translocation (15). However, despite this indirect evidence that they reduce iron, measurements of iron reductase (FeRed) activity by cells of this premier iron-accumulating bacterium are needed. In this chapter, we report the cellular locations and biochemical characteristics of FeRed activity detected in cell fractions of A. magnetotacticum strain MS-1 and NM-1A grown under conditions appropriate for magnetite formation.

Materials and Methods

Bacterial strains and growth conditions. Aquaspirillum magnetotacticum strain MS-1 (ATCC 31632) and NM-1A (15) were batch cultured microaerobically in magnetic spirillum growth medium (13).

Preparation of cell extracts. Cells were harvested (14) and washed once in 10 mM Tris (2-amino-2-hydroxymethylpropane-1,3-diol) buffer, pH 8.0. Washed cells suspended in 6-8 ml of buffer were disrupted by two passes through a French pressure cell at 16,000 lb/in^2. Undisrupted cells and debris were removed by centrifugation (10,000 x g, 15 min, 4°C). The cell membrane and soluble fractions were obtained from the supernatant fluids by ultracentrifugation (100,000 x g, 1 h, 4°C). Membranes in the pellet fraction were suspended in 1-2 ml of buffer and stored on ice. Periplasm was obtained by the freeze-thaw method (14) and cytoplasm was subsequently released from thawed cells by disruption in a French pressure cell. Outer and cytoplasmic membranes were separated with Triton X-100 (Sigma Chemical Co., St. Louis, MO) as previously described (13). Samples were immediately assayed for FeRed activity.

Protein concentrations were determined by the method of Lowry et al. (10).

Iron reductase assay. Iron reduction by cell extracts was measured with ferrozine (ϵ_{562}= 28,000 M^{-1} cm^{-1}) as described by Dailey and Lascelles (4). Assays were performed anaerobically in Thunberg cuvettes at 25°C in a Beckman Instruments DU-8 spectrophotometer equipped for kinetic analysis. Reaction mixtures (final volume of 2.0 ml) consisted of: 10 mM Tris-acetate buffer (pH 8.1) containing 10 % (v/v) glycerol and 10 µg/ml phenylmethylsulfonyl fluoride; 0.8 mM β-NADH; 0.5 mM ferrozine; 0.2 mM ferric-citrate; and 10 uM flavin mononucleotide (FMN). Cell extract (0.5 - 2.0 mg protein) was placed in the sidearm and the contents sparged with O_2-free N_2 for 5 min. The reaction was initiated by mixing extract with the assay mixture. Apparent Km and Vmax values were calculated from Lineweaver-Burke plots obtained with a minimum of three substrate concentrations.

Iron chelates. Ferric chloride (4 mM) was combined with each of the following chelators (4 mM each in Milli-Q water): quinic acid (Sigma Chemical Co, St. Louis, MO), 2,3-dihydroxybenzoic acid, (DHB, Aldrich Chemical CO., Milwaukee, WI), acetohydroxamic acid (Aldrich Chemical Co., Milwaukee, WI), and deferrioxamine B (a generous gift from Ciba-Geigy Corp., Summit, NJ).

Chemicals. β-NADH, β-NADPH, FMN, FAD, rotenone, antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) were purchased from Sigma Chemical Co.

Results and Interpretations

Distribution of FeRed activity. Of the total FeRed specific activity detected in strain MS-1 and NM-1A, 77 and 64 %, was in the cell

periplasmic fraction, respectively (Table 3). Approximately 20 (strain MS-1) to 35 % (strain NM-1A) of the total FeRed activity was present in the cytoplasm and 3 %, was detected in combined membrane fractions (Table 3). The outer membrane fraction contained approximately 1 % of the total activity and no activity was detected in the cytoplasmic membrane fraction. Specific activity values of soluble or membrane-associated FeRed of A. magnetotacticum (Table 3) were similar to those reported for other organisms (1, 2, 4, 9, 11)

The preponderant distribution of FeRed activity in periplasm could be important in iron assimilation (13), in formation of the periplasmic hemes detected previously in this organism (12), in substrate dissimilation (15) or a combination of these. Cytoplasmic FeRed of A. magnetotacticum could be associated with iron transport, or with reduction of intracellular ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$), to magnetite ($Fe_2O_3 \cdot FeO$) (5).

Biochemical properties of FeRed. We detected FeRed activity in soluble fractions from cells cultured over the range 0 to 40 μM added iron (data not shown) indicating that this enzyme was constitutively produced. Specific activity of either soluble or membrane-associated FeRed in strain MS-1 cells was at least 2-fold higher with B-NADH than with β-NADPH as the reductant (Table 4). Succinate did not serve as a reductant for either soluble or membrane-associated FeRed (Table 4). Flavins have been shown to stimulate FeRed activity (8, 9, 11). The activity of both soluble and membrane-associated FeRed of strain MS-1 cells was enhanced by adding FMN or FAD to the assay mixture (Table 5). Activity was not detected in cell combined (outer plus cytoplasmic)

membrane fractions in the absence of added flavins. Reduction was not observed with either 0.8 mM β-NADH (or β-NADPH) or 10 μM FMN (or FAD) added to the assay mixture in the absence of enzyme. Activity was completely destroyed by boiling cell fractions.

Preferential use of β-NADH over β-NADPH and the stimulatory effect of flavins are characteristics shared by FeRed in Neisseria gonorrhoeae (8), Rhodopseudomonas sphaeroides (11), Pseudomonas aeruginosa (2), and Agrobacterium tumefaciens (9).

As with other bacteria (4, 9), the FeRed activity we measured was totally and reversibly inhibited by oxygen (data not shown). Inhibition was relieved by sparging mixtures with O_2-free N_2 or by adding 2-mercaptoethanol (0.1 % v/v) to the assay mixture. Lodge et al. (9) have reported bacterial FeRed inhibition by sulfhydryl binding agents such as mercuric or cadmium chloride and N-ethylmaleimide. Mercuric chloride (1 mM) completely abolished iron reduction by the soluble cell fraction of strain MS-1. These results suggest that reduced disulfide bonds, which can be disrupted by O_2, and uncomplexed sulfhydryl groups may be necessary for soluble FeRed activity in this bacterium.

Respiratory inhibitors HQNO, antimycin A or rotenone (4 μM each) inhibited iron reduction by combined membrane fractions of A. magnetotacticum MS-1. The rates were 11 % or less, of those of untreated controls. Because the cytoplasmic membrane fraction exhibited no FeRed activity, our results indicate that the FeRed activity we measured was not associated with cell respiration. These results are at variance with those of Short and Blakemore (15) who

demonstrated that intact cells of this strain (cultured similarly) carry out iron respiration-driven proton translocation; a process which was inhibited by respiration inhibitors. Although some organisms apparently use respiratory chain components for iron reduction (4, 7), recent evidence was presented showing that iron reduction by Escherichia coli K12 cells was not respiration-linked (16). Differences between our work and that of Short and Blakemore (15) may relate to our use of cell membranes rather than intact cells.

The specific activity of the membrane FeRed of strain MS-1 with ferric-DHB was 4-fold higher than with other ferric chelates tested. Except for this effect, we observed no differences in the specific activity of either periplasmic, cytoplasmic or membrane reductases of strain MS-1 with any of the six iron chelates tested (those listed in Table 6). Cox (2) correctly predicted that P. aeruginosa cells possessed two different soluble iron reductases based, in part, upon measured differences in specific activity of extracts with two iron chelates. By this reasoning, because we observed similar activity with diverse chelates, A. magnetotacticum may contain one soluble FeRed able to process iron uncomplexed or bound to a variety of chelates.

Apparent Km and Vmax values. Apparent Km values of soluble (periplasmic and cytoplasmic) FeRed from strain MS-1 ranged from 18 μM for ferric quinate to 212 μM for ferric citrate (Table 6). The Vmax values ranged from 9 nmol · min $^{-1}$ · mg protein^{-1} for ferric DHB to 52 nmol · min^{-1} · mg protein^{-1} for ferric citrate (Table 6). These data indicate that A. magnetotacticum cells are able to reduce uncomplexed iron or iron complexed to citrate, phenolates or to primary or

secondary hydroxamates. Ferric quinate appeared to be an especially suitable iron substrate. This may reflect the culture history of cells because ferric quinate is the iron source in our growth medium.

Conclusions

Our data show that FeRed activity of A. magnetotacticum was predominately located in the periplasm. The enzyme reduced uncomplexed iron or iron complexed to any of several chemically different chelators and was constitutively produced over the range of culture iron from 0 to 40 μM. Enzyme activity required β-NADH, was enhanced by flavins, was reversibly inactivated by oxygen, and was unaffected by a selection of respiratory inhibitors.

Cells of mutant strain NM-1A accumulate substantial amounts of iron as ferrihydrite but not magnetite (5). We did not observe substantial differences between the magnetic and non-magnetic strains with regard to the location and specific activity of FeRed. Our results corroborate earlier findings (D.A. Bazylinski, Ph.D. thesis, University of New Hampshire, Durham, 1984) and suggest that iron reducing ability is not a factor explaining the inability of NM-1A cells to produce magnetite.

In some organisms, iron is apparently released from ferrisiderophores by reduction (1-4, 7-9, 11, 16). However, in few studies have comparisons been made of FeRed activity in separated periplasmic, cytoplasmic and membrane cell fractions. Iron accumulates intracellularly in both wild type strain MS-1 and non-magnetic mutant strain NM-1A as magnetite or ferrihydrite, respectively. Because it does not accumulate at the cell surface, transport through cell

boundary layers and periplasm cannot be a rate-limiting step in iron aquisition by these strains. Abundant periplasmic iron reductase activity could promote iron transport and prevent accumulation of surficial ferric oxyhydroxides in this motile spirillum.

Acknowledgments

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Table 3. Cellular distribution of iron reductase activity in *Aquaspirillum magnetotacticum*.

Cell Fraction	Activity in strain: MS-1 % Total	MS-1 Sp. Act.[a]	NM-1A % Total	NM-1A Sp. Act.
Periplasm[b]	77	25	64	37
Cytoplasm	20	9	35	7
Membranes[c]	3	2	3	1

[a] nmol · min^{-1} · mg $protein^{-1}$

[b] Obtained by freeze-thaw method (14).

[c] Includes outer plus cytoplasmic membranes.

Assay conditions are described in Materials and Methods section.

Table 4. Effect of reductants on iron reductase activity of *Aquaspirillum magnetotacticum* strain MS-1.

Reductant (mM)	Specific activity[a] in: Soluble fraction	Membrane fraction[b]
NADH (0.8)	8.2	2.4
NADPH (0.8)	2.0	1.0
Succinate (4.0)	N.D.A.[c]	N.D.A.

[a] nmol Fe(II) formed · min^{-1} · mg $protein^{-1}$

[b] Includes outer and cytoplasmic membranes.

[c] No detectable activity.

Assay conditions are described in Materials and Methods section.

Table 5. Effect of flavins on iron reductase activity of *Aquaspirillum magnetotacticum* strain MS-1.

Flavin (10 μM)	Specific activity[a] in: Soluble fraction	Membrane fraction[b]
None	1.2	N.D.A.[c]
FMN	8.9	1.6
FAD	11.8	2.4

[a] nmol Fe(II) formed · min^{-1} · mg $protein^{-1}$

[b] Includes outer plus cytoplasmic membranes.

[c] No detectable activity.

Table 6. Apparent Km and Vmax values of Aquaspirillum magnetotacticum strain MS-1 soluble iron reductase with various iron compounds.

Iron compound	Km[a]	(r)[b]	Vmax[c]
Ferric-quinate	18	(0.98)	22
Ferric-DHB	26	(0.99)	9
Ferric-acetohydroxamate	56	(0.98)	16
Ferric-chloride	105	(0.99)	38
Ferric-ferrioxamine B	154	(0.99)	15
Ferric-citrate	212	(0.99)	52

[a] μM.

[b] Km, r (correlation coefficient) and Vmax values were calculated from Lineweaver-Burke plots of enzyme activity at three or more substrate concentrations.

[c] nmol Fe(II) formed · min^{-1} · mg $protein^{-1}$.

CHAPTER FOUR

Chapter Four

Freezing and Thawing Cells of Aquaspirillum magnetotacticum Selectively Releases Periplasmic Proteins.

Abstract

Cells of the Gram-negative bacterium Aquaspirillum magnetotacticum, when suspended in buffer, frozen and thawed, produced pink-orange supernatant fluid. The fluid contained 2.0 % or less of total extractable outer membrane component 2-keto-3-deoxyoctonate and of the cytoplasmic membrane marker succinic dehydrogenase. Electrophoretic banding patterns and difference spectra of proteins and hemoproteins released by freezing and thawing cells were distinct from those of membrane-associated and similar to those of periplasmic substances obtained by applying conventional fractionation methods to this organism.

Freezing and thawing is undoubtedly important in defining indigenous bacterial populations of soils in temperate regions. Morley et al. (11) observed a 40-60 % decrease in bacterial viability in sandy loam soil as a direct result of freezing and thawing. Freezing and thawing is known to have a profound effect on bacterial cells, and is often used as a pretreatment to cell disruption (18, 19). Responses of Gram-negative cells depend upon the cell genotype (3), the menstrum they are suspended in and the freezing and thawing rates (4). Outer sheath material from an oral spirochete has been isolated by freezing and thawing (10). Calcott and MacLeod (4) found that frozen and thawed

lactose-limited Escherichia coli cells, released considerable amounts of the periplasmic enzyme cyclic phosphodiesterase, but not the cytoplasmic enzyme glucose-6-phosphate dehydrogenase. A small, constant quantity (10 to 15 % of total activity) of B-galactosidase (normally cytoplasmic) released was attributed to a possible periplasmic form of this enzyme.

Periplasmic substances of E. coli have been separated from other cellular components by means of osmotic shock or spheroplast formation (12). Ames et al. (1) demonstrated selective release of periplasmic proteins of E. coli cells treated with chloroform.

Freezing and thawing cell suspensions of Aquaspirillum magnetotacticum strain MS-1 in 10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (Hepes) buffer (pH 7.4) or 10-50 mM potassium phosphate buffer (KPB) caused the release of soluble c_{551}-type hemoproteins (W. O'Brien, M.S. thesis, University of New Hampshire, Durham, 1982; 15). This freezing and thawing method (F/T) did not disrupt overall helical cell morphology. The objective of our study was to compare F/T with other cell fractionation methods applied to this organism to determine the cellular origin of the substances released including the soluble c_{551}-type hemoprotein. This method also allowed us to partially purify this hemoprotein. Periplasmic soluble c-type hemoproteins of unknown function have been detected in Alcaligenes eutrophicus (17), Aquaspirillum itersonii (6), Paracoccus denitrificans (8), and in Haemophilus parasuis (13). (Portions of this work have been reported [Paoletti, Short and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I6, p. 166]).

Denitrifying cells of Aquaspirillum magnetotacticum MS-1 (ATCC 31632) were batch cultured with chemically defined medium (2) microaerobically at a dissolved oxygen tension (d.o.t.) less than 1 % of saturation. Cells were harvested by filtration (15) when they reached a density of $4 \times 10^8 \cdot ml^{-1}$. Cells were washed once by centrifugation (8,000 x g, 30 min, 5°C) in at least 10 pellet volumes of 50 mM KPB (pH 6.8) or 10 mM Hepes buffer (pH 7.4). Cells from a single 40 l culture were suspended in 100 ml of KPB or Hepes buffer and equal portions were fractionated by the procedures described below.

The F/T technique consisted of storing washed, resuspended cells at -20°C overnight. The freezing rate was $0.7°C \cdot min^{-1}$. The sample was thawed at room temperature and cells were pelleted by centrifugation (10,000 x g, 15 min, 5°C). The pink-orange supernatant fluid was clarified by ultracentrifugation (100,000 x g, 1 h, 5°C) and concentrated by membrane dialysis (Spectrapor, 6,000-8,000 mol wt cutoff, Spectrum Medical Industries, Inc., Los Angeles) on a bed of polyethylene glycol (solid flake, 20,000 mol wt, J. T. Baker Chemical Co., Phillipsburg, New Jersey) at 4°C.

Periplasmic proteins were obtained by two methods: osmotic shock (12), or chloroform extraction (1). The Schnaitman method (19) was also used to separate outer membrane proteins, cytoplasmic membrane proteins and soluble (cytoplasm and periplasm) proteins. Cells were disrupted in a French press (10,000 p.s.i.) prior to treatment with 2 % (v/v) Triton X-100 and 10 mM $MgCl_2$ in 10 mM Hepes buffer (pH 7.4). Each fraction was dialyzed overnight at 4°C against Hepes buffer prior to analysis.

The relative activity of succinic dehydrogenase (SDH), an integral enzyme of the cytoplasmic membrane (5, 7), and the concentration of 2-keto-3-deoxyoctonate (KDO), a constituent of outer membrane lipopolysaccharide were used as indices of the purity of cell fractions (9). Proteins and molecular weight standards (Bio-Rad, Richmond, California) were solubilized and separated by SDS-PAGE (16) and stained with silver (14). Room temperature dithionite-reduced minus air-oxidized difference spectra were performed with the soluble protein fractions as previously described (15). The ability of cells to survive F/T was evaluated by a standard plate assay. Thawed cells were quantitatively diluted and aliquots prepared as pour plates in semi-solid medium in triplicate. Plates were incubated one week at room temperature microaerobically. Colony counts were compared to those of control (non-F/T) cells plated similarly.

Supernatant fluids obtained by F/T contained 1.3 % of the total SDH activity detected and 2.0 % of the total KDO recovered (Table 7). These results suggest that F/T did not markedly disrupt either the outer or inner cell membranes with attendant release of these markers. Soluble fractions obtained by chemical treatment (chloroform or lysozyme-EDTA) or mechanical disruption (French press) of strain MS-1 cells had comparable proportions of total detectable SDH activity and KDO (Table 7). Most (87 %) of the total SDH activity and 90 % of the total KDO recovered were in the cytoplasmic and outer membrane fractions of strain MS-1 cells, respectively (Table 7). Electrophoretograms of each soluble fraction (Fig. 11, lanes 4-7) exhibited similar protein banding patterns. In each of these

fractions, more than 60 proteins were evident including several major proteins with molecular weights ranging between 28,000 and 85,000 daltons. Four proteins with apparent molecular weights of 29,000, 41,000, 44,500 and 45,000 dalton were unique to the periplasm (Fig. 11, lanes 4-7). The cytoplasmic membrane (Fig. 11, lane 3) contained three major proteins (16,500, 56,000 and 85,000 daltons) also present in the periplasmic fraction. The outer membrane (Fig. 11, lane 2) and periplasmic fractions (Fig. 11, 4-7) contained few proteins in common.

Soluble fractions obtained by F/T, chloroform treatment, osmotic shock or French press disruption of strain MS-1 cells contained substances with absorption spectra (Fig. 12) typical of c_{551}-type hemes (maxima at 419, 522 and 551 nm). Spent growth medium and cell wash fluids of A. magnetotacticum concentrated 100-fold did not contain detectable quantities of protein or c-type hemoproteins.

The effects of F/T on strain MS-1 cells were evaluated by the plate assay and electron microscopy. Only 1-7 % of the number of control (non-F/T) cells were recovered as colony forming units following F/T. Survivors were magnetotactic. F/T treated cells when negatively stained with uranyl acetate and observed by electron microscopy lacked flagella but appeared otherwise structurally intact as compared to control cells. They retained their helical morphology and did not form spheroplasts or show blebbing.

Our results indicate F/T provides a rapid, simple, reproducible method of selectively releasing periplasmic substances including the soluble c_{551}-type hemoproteins from A. magnetotacticum without recourse to chemical treatments.

We have applied F/T to cells of A. itersonii and Azospirillum lipoferum and obtained spectral evidence for release of c-type hemoproteins from these organisms as well (data not shown). Recently, F/T was applied to cells of nine genera of Gram-negative bacteria. The method was found to be comparable to the chloroform method (1) for the release of periplasm (B. E. Eribo, S. D. Lall and J. M. Jay, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I152, p. 197).

F/T had several advantages over conventional techniques used to obtain periplasm from A. magnetotacticum. These include (i) the absence of chemical treatment such as with lysozyme, chloroform, toluene or EDTA, (ii) the rapid and selective recovery of periplasmic substances including enzymes, and (iii) lack of apparent gross cell damage. This method, if generally applicable to other Gram-negative species, would prove useful in obtaining periplasm with minimal cell handling; as with pathogens, for instance.

Acknowledgments

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Table 7. Membrane markers in cell fractions of *A. magnetotacticum*.

Fractionation Method	Total KDO (mg)	Total SDH[a] (units x 10^{-2})
Triton X-100/$MgCl_2$ (ref. 19)		
OMP	1330	30
Periplasm/Cytoplasm	6	0.9
CMP	149	200
Chloroform (ref. 1)		
Periplasm	11	0.9
Osmotic shock (ref. 12)		
Periplasm	16	2.0
Freeze/Thaw (this study)		
Periplasm	20	5.0

[a] μmoles cytochrome c reduced $\cdot$ mg^{-1}protein $\cdot$ min^{-1}.

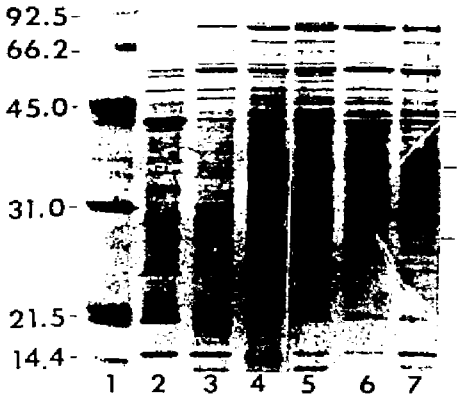

FIG. 11. Silver stained SDS-PAGE of Aquaspirillum magnetotacticum cell proteins. Lane 1, molecular weight standards in kdal. Lane 2, outer membrane fraction. Lane 3, inner membrane fraction. Lane 4, periplasm/cytoplasm fraction. Lane 5, proteins obtained by the freeze/thaw method. Lane 6, proteins released by osmotic shock. Lane 7, proteins obtained with lysozyme-EDTA treatment. Each lane contained 3.5 μg protein. Dashes indicate proteins restricted to the periplasm.

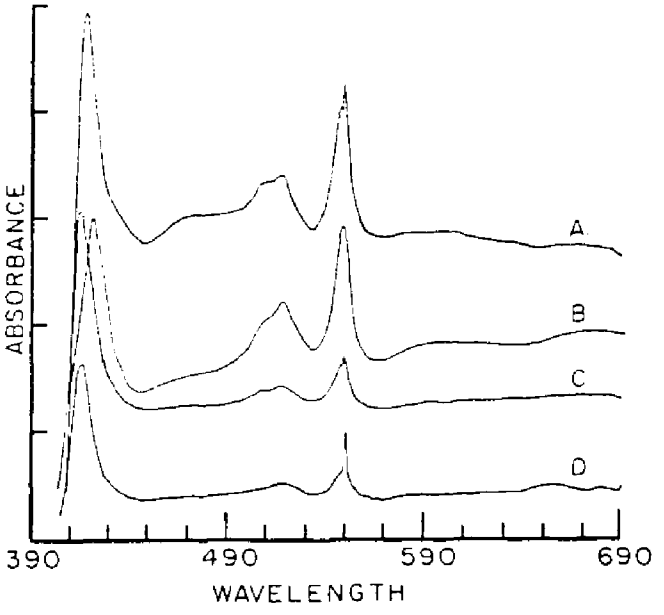

FIG. 12. Reduced minus oxidized difference spectra of soluble proteins of Aquaspirillum magnetotacticum obtained by fractionation using: A. Freezing/thawing method (0.7 mg protein; absorbance divisions = 0.045). B. Schnaitman procedure (0.9 mg protein; absorbance divisions = 0.260). C. Chloroform treatment (0.04 mg protein; absorbance divisions = 0.045). D. Osmotic shock (0.04 mg protein; absorbance divisions = 0.010).

CHAPTER FIVE

Chapter Five

Periplasmic c-type Hemoproteins of Aquaspirillum magnetotacticum.

ABSTRACT

Denitrifying cells of Aquaspirillum magnetotacticum strain MS-1 contain soluble forms of c- and cd_1-type hemoproteins. Soluble c_{551} of strain MS-1 occurred in two forms; a 17,000 dalton free form, and together with d_1 as a component of an 85,000 dalton complex (nitrite reductase).

INTRODUCTION

Due to their hydrophilic nature, c-type hemoproteins are often found in the Gram-negative cell periplasm or loosely associated with the cytoplasmic membrane. They, along with other respiratory components, serve a role in energy transduction (4, 5, 8). Soluble cytochromes include c_{551}- and cd_1-types. The latter contains a dihydroporphyrin or chlorin moiety in lieu of a heme group (5, 8, 12). Cytochrome cd_1 has been shown to function as a cytochrome oxidase and in nitrite reduction (8).

Cells of the magnetite (Fe_3O_4)-producing spirillum A. magnetotacticum are non-fermentative obligate microaerophiles which oxidize organic acids. They denitrify microaerobically, (d.o.t. at 1 % of saturation) but will not grow anaerobically with NO_3^-. While denitrifying, cells respire using NO_3^- and O_2 as terminal oxidants simultaneously (1, 13). The cytochrome complement of these cells include a-, a_1-, b-, c-, cd_1- and o-type hemes (10). Large quantities

of c- but not a- or b-types cytochromes partition with cell soluble fractions.

The results presented here include further characterization of soluble c-type hemoproteins of this magnetotactic spirillum by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and spectral analysis.

MATERIALS AND METHODS

Organism and culture conditions. Aquaspirillum magnetotacticum strain MS-1 cells were cultured in a chemically defined growth medium, MSGM (2) in glass carboys under microaerobic conditions (initial headspace O_2, 0.2 to 2 % atmosphere). Cells were harvested by continuous flow centrifugation or by filtration through 0.45 um microporous membranes in a Millipore Pellicon Cassette filtration system (Millipore Corp., Medford, MA.).

Extraction of soluble cytochromes (c + cd_1). Washed cells (ca. 3 x 10^{11}) in 6 ml cold potassium phosphate buffer KPB (pH 6.8) were frozen at -12° C overnight. The thawed cells were centrifuged (8,000 x g, 20 min, 5°C) and the supernatant fluids were further clarified by centrifugation (32,000 x g, 30 min, 5°C). The pink supernatant fluid was placed in a dialysis bag (Spectrapor #1, 6,000-8,000 m.w. cutoff, Spectrum Medical Industries, Inc., Los Angeles, CA.) and concentrated to one-sixth its volume using polyethylene glycol (J.T. Baker, solid flake, 20,000 m.w.) by the method of Cliver (3). The sample was then dialyzed overnight at 4°C against KPB (pH 6.8) and examined for cytochromes.

Soluble hemoproteins were also prepared from cells disrupted at

4°C with a French pressure cell. Cell debris was removed by centrifugation (4,300 x g; 15 min; 4°C) and the supernatant fluid was clarified by ultracentrifugation (200,000 x g; 1 hr; 4 °C). The resulting light brown supernatant material was applied to a DEAE-cellulose (Sigma Chemical Co., St. Louis, MO) column equilibrated with 20 mM sodium acetate (pH 6.0). Amber colored material containing the c-type hemes eluted with the void volume and was collected for spectral analysis. Protein was measured using the Lowry et al. (7) method with bovine serum albumin as a standard.

Absorbance spectra. Room temperature difference spectra were measured in a Beckman Instruments DU-8 UV/VIS spectrophotometer equipped for wavelength scanning. This single beam recording spectrophotometer stored the reference spectrum in a microprocessor RAM and subsequently subtracted it from that of the sample to obtain a difference spectrum.

Reduced minus oxidized (red-ox) difference spectra were obtained by subtracting the spectrum of air oxidized sample from that of the same sample following either chemical (sodium dithionite) reduction.

SDS-PAGE. Cytochromes released by the freeze/thaw method were concentrated as described above, solubilized at room temperature and separated on a 1.5 mm SDS-polyacrylamide slab gel using the buffer system of Laemmli (6). The ratio of acrylamide to bis-acrylamide was 30:2.7 and the concentration of acrylamide in the stack and separating gels was 4 and 17 %, respectively. Each gel contained concentrated samples (each 63 µg protein), molecular weight standards (Bio Rad, Richmond, CA), and horse cytochrome c (Type II-A, Sigma, St. Louis, Mo.). Proteins migrated through the stacking and separating gels at a

constant current of 20 (2.5 h) and 40 mA (2.0 h), respectively. Preparative gels (1.5 mm) contained 17-25 mg protein. These gels consisted of a 3.5 cm, 4 % acrylamide stacking gel and a 7.0 cm, 12 % acrylamide separating gel. Proteins migrated through the stacking and separating gels at a constant current of 10 and 20 mA, respectively. Gels were observed unstained and after staining with diaminobenzidine to reveal c-type cytochromes (9), or with Coomassie Blue R-250 to reveal proteins.

Results and Discussion

Suspensions of denitrifying cells in KPB (pH 7.0), when frozen overnight, thawed and centrifuged, yielded pink supernatant wash fluids with spectral characteristics of c- and d_1-type hemes (Fig. 13). The absorption band in the vicinity of 551 nm was split suggesting the presence of more than one c-type. Proteins in these wash fluids, when concentrated and separated using SDS-PAGE, included a pink and a brown band of apparent mol. wt. 17,000 and 85,000 daltons, respectively. Each of these exhibited peroxidase activity typical of c-type hemes (5, 9).

After treatment of freeze/thaw fluids with DEAE-cellulose (see above), well defined red-ox maxima attributable to the d_1 (419, 468, and 616 nm) and c (419, 522, and 551 nm) hemes of nitrite reductase were observed (Fig. 14). Additional maxima characteristic of a second c-type heme (549 and ca. 522 nm) were also present (Fig. 14).

When solubilized at 25°C, electrophoresed, and subsequently eluted from the unstained gel, the pink material displayed spectral characteristics (absorption maxima at 412 and 551 nm) of a c-type heme.

The 85,000 dalton brown band produced by preparative SDS-PAGE of 17-25 mg of proteins released by freeze/thaw was resolved by prolonged electrophoresis into a green (apparent mol. wt. of 83,000 daltons) and a pink (apparent mol. wt. of 81,000 daltons) band (not shown). Spectra obtained using material from the green band had (dithionite) red-(persulfate) ox absorption maxima at 415 nm and 551 nm, as expected of heme c and at 468 nm and 625 nm as expected for the d_1 chlorin of cytochrome cd_1 (Fig. 15).

Freezing and thawing released periplasmic proteins of this organism but not proteins in the cytoplasmic membrane as determined from cell fractionation studies and assay of succinic dehydrogenase (11). Thus, c-type hemes were selectively released by this treatment and by general cell disruption could not explain their abundance as soluble hemoproteins.

Collectively, our results indicate the presence of soluble c-, and cd_1-type hemes in A. magnetotacticum. Further research is required to elicudate the function of these periplasmically abundant proteins.

Acknowledgments

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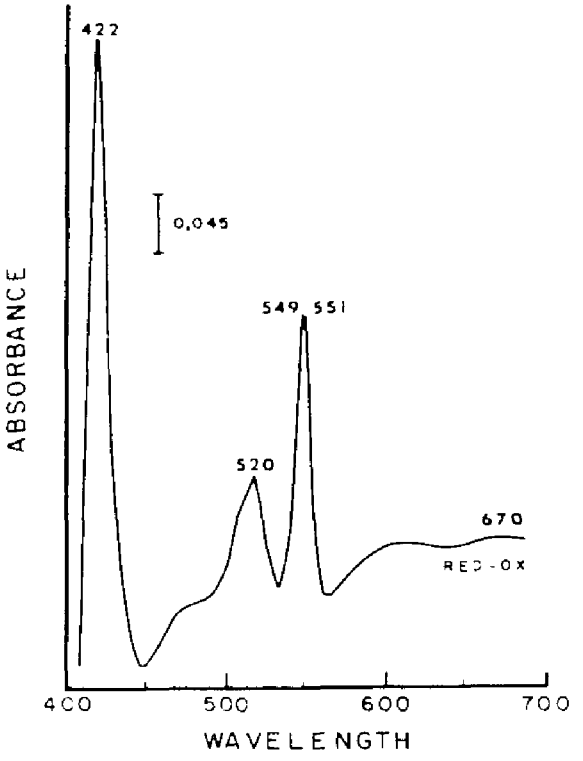

FIG. 13. Room temperature reduced minus oxidized difference spectrum of soluble proteins released by freezing and thawing A. magnetotacticum MS-1 cells. The protein concentration was 3.6 mg ml^{-1}.

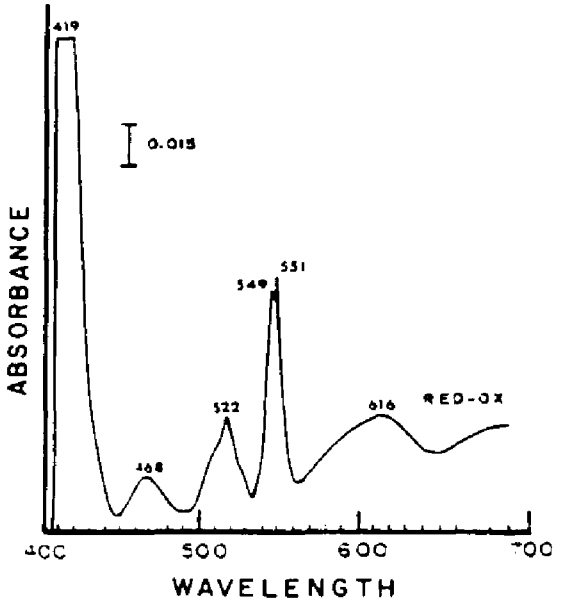

FIG. 14. Room temperature reduced minus oxidized difference spectrum of soluble proteins released by freezing and thawing A. magnetotacticum MS-1 cells and partially purified by treatment with DEAE-cellulose. The protein concentration was 4.0 mg • ml^{-1}.

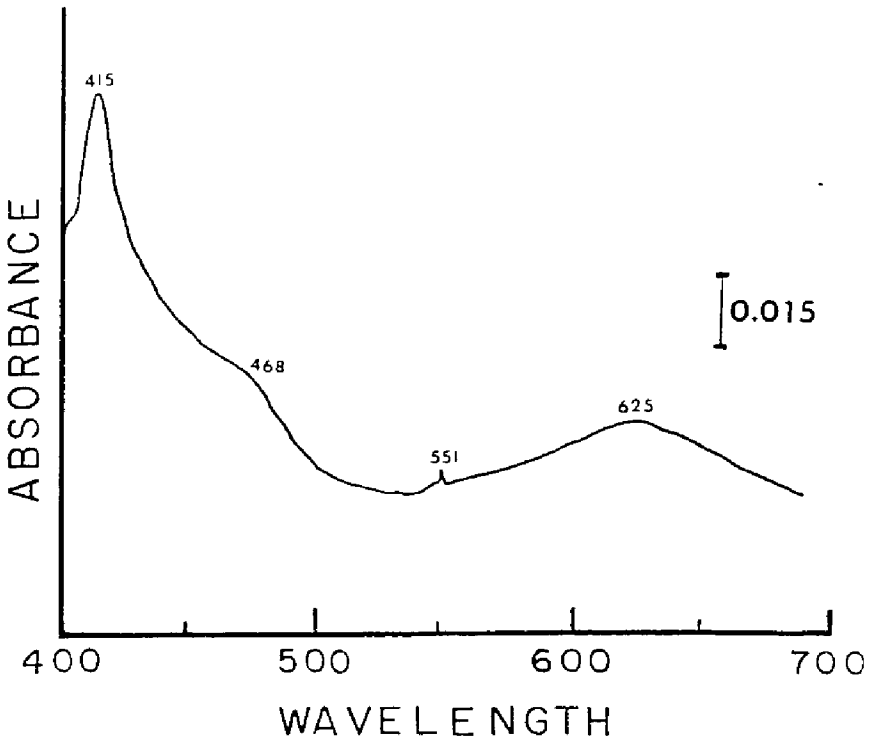

FIG. 15. Room temperature dithionite reduced minus persulfate oxidized spectrum of 83,000 dalton soluble green protein of Aquaspirillum magnetotacticum excised from a preparative SDS-polyacrylamide gel.

CHAPTER SIX

Chapter Six

Antigenic Relatedness of a Periplasmic c-type Cytochrome and an Outer Membrane Protein in Aquaspirillum magnetotacticum.

Abstract

Cells of Aquaspirillum magnetotacticum strain MS-1 each possess a 17,000 dalton outer membrane protein antigenically similar to a periplasmic cytochrome c_{551} of equal mass but antigenically distinct from the 81,000 dalton periplasmic heme c of cytochrome cd_1.

Introduction

The Gram-negative cell outer membrane is a lipid bilayer interspersed with proteins and serves as a barrier to some substances such as antibiotics while allowing the entry of nutrients (2, 9). In addition to its involvement with nutrient transport, the cytoplasmic membrane, also contains components of energy transduction including flavoproteins, quinones and cytochromes. Cytochromes are characteristically present in, and have been used as markers for, the cytoplasmic membrane. However, many bacteria also contain soluble c-type hemoproteins in their periplasm (6, see refs in 8).

Microaerobically denitrifying cells of Aquaspirillum magnetotacticum MS-1 possesses a-, a_1-, b-, c-, cd_1- and o-type cytochromes (6). More than 70 % of the c-types were soluble and appeared as pink bands on unstained preparative sodium dodecyl sulfate-polyacrylamide gels. The c_{551}-type cytochrome had a molecular mass of 17,000 daltons (17 kdal) and was both membrane bound and soluble. A second hemoprotein with a molecular mass of 81 kdal was associated with

the d_1 chlorin of cytochrome cd_1 (6).

A. magnetotacticum strain MS-1 cells also produced, over a wide range of culture iron concentrations, a 17 kdal outer membrane protein (OMP) which comprised 25 % of the total OMPs detected (L.C. Paoletti, M.S. thesis, University of New Hampshire, 1984). The possibility that this OMP and the 17 kdal c_{551} hemoprotein were one and the same was intriguing because bacterial outer membranes were not known to include cytochromes. Here we report that this periplasmic 17 kdal cytochrome c_{551} and the OMP of similar mass in denitrifying cells of A. magnetotacticum are antigenically similar.

Materials and Methods

Cell strain and growth conditions. Denitrifying cells of A. magnetotacticum strain MS-1 (ATCC 31632) were grown in batch cultures microaerobically with magnetic spirillum growth medium (1). Cells were harvested (8) and washed once by centrifugation (7,000 x g, 15 min, 4°C) in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer, pH 7.4. Washed cells suspended in Hepes were either fractionated immediately or stored at -70°C.

Cell fractionation. Soluble and membrane proteins of strain MS-1 cells were separated by the Triton X-100/$MgCl_2$ treatment of Schnaitman (10). This method produced three cellular fractions (7): the outer membrane (OM), the cytoplasmic membrane (CM) and soluble proteins derived from both the periplasm and cytoplasm (P+C). Periplasmic proteins (P) obtained by the freeze-thaw method (8) were concentrated and dialysed as previously described (6). Proteins were quantified (4) and stored

at -70°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prior to electrophoresis, proteins were solubilized in reducing buffer (3) at 25°C or 100°C. Proteins (30 μg) were then separated by SDS-PAGE using the procedure and buffers of Laemmli (3). A 4 % stacking and 12 or 17 % separating gels were used. Proteins and molecular weight standards (Bio-Rad, Richmond, CA) were visualized with Coomassie blue. Hemoproteins were detected on gels by staining with diaminobenzidine for peroxidase activity (5).

Antigens and antisera. Periplasmic proteins (17-25 mg) were solubilized in reducing buffer at 25°C and separated in an 1.5 mm preparative SDS-PAGE. Two pink (17 and 81 kdal) and one green band (83 kdal) were observed on unstained gels. The 17 and 81 kdal pink bands were separately used as antigens. These hemoproteins were sliced from the gel and homogenized with 0.5 ml of Freund's complete adjuvant and 0.5 ml phosphate buffered saline, pH 7.2 (PBS). This mixture was subcutaneously injected into a five week old New Zealand White rabbit at four different sites on the back. Three antisera were made. For "anti-17" serum, a rabbit was immunized with the 17 kdal protein on day one and seven and bled on day 17. "Anti-81" antiserum was prepared by immunizing a second rabbit with the 17 kdal protein on day one, challenging with the 81 kdal protein on day seven and bleeding on day 18. This animal was again rechallenged with original (17 kdal) immunogen on day 46 and serum collected on day 50. Antiserum obtained on day 50 was designated "anti-81+17". Gamma globulin fractions precipitated from sera with cold saturated ammonium sulfate (pH 7.8),

were recovered by centrifugation (1800 x g, 30 min, 5°C. The pellet was resusupended in PBS and dialysed against PBS overnight at 4°C. The immunoglobulin fractions from normal and immune sera were used to probe cellular fractions of A. magnetotacticum.

Immunoblot. Proteins were electrophoretically transferred from a SDS-polyacrylamide gel to a nitrocellulose membrane in a Trans-blot cell (Bio-Rad, Richmond, CA) at a constant current of 175 mA at 5°C for 17 to 24 h as described by Towbin (11). Membranes were incubated with serum diluted at 1/100 as described by the Bio-Rad Immun-Blot instruction manual. Whole cells and anti-17 serum were also used to determine whether the 17 kdal OMP is exposed on the outer surface of the outer wall of strain MS-1. This assay was performed with a sampling manifold (model 1225, Millipore, Bedford, MA) modified to allow retainment of fluids during incubations by plugging each hole underneath the support plate. Active cells ($2.5 \times 10^9 \cdot \text{filter}^{-1}$) were vacuum filtered onto 0.45 μm GA-6 membranes (Gelman Sciences, Ann Arbor, MI) and rinsed once with 5 ml of PBS. Cells were then incubated overnight on a rotary shaker (100 rpm) at room temperature with 1-2 ml of either anti-17 serum, normal rabbit serum (diluted 1 to 9 with PBS) or PBS. Cells were again filtered and rinsed with 4 ml of PBS prior to the addition of protein A-horseradish peroxidase conjugate (1 ml) for 2 h at room temperature without shaking. Cells on filters were then rinsed with 4 ml PBS prior to the addition of 1 ml HRP color developer solution. The reaction was allowed to proceed for 0.5 h.

Results

Soluble proteins. When separated by preparative SDS-PAGE, 8-10

pigmented periplasmic proteins were evident on unstained gels. Most striking were the pink 17 and 81 kdal and green 83 kdal protein bands (Fig. 16). The 81 kdal pink and the 83 kdal green bands comprised the heme and chlorin components, of cytochrome cd_1 (nitrite reductase), respectively as determined from spectral analysis (6; see also Chapter Five). Periplasmic proteins were examined for peroxidase activity, typical of c-type hemoproteins (5). When solubilized at 25°C prior to electrophoresis, proteins with molecular mass of 10, 17, 23, and 51 kdal had peroxidase activity (Fig. 17, lane 1). The 17 kdal pink protein was absent from samples solubilized at 100°C but a 20 kdal protein with peroxidase activity, not observed in samples solubilized at 25°C, was present (Fig. 17, lane 2). A 30 kdal protein present in the cytoplasmic membrane and outer membrane fractions was weakly reactive with the peroxidase stain (not shown).

Immunoblots. Normal rabbit immunoglobulins were unreactive with proteins of A. magnetotacticum cells (Fig. 18, panel A). "Anti-17" serum reacted with 12-15 soluble proteins (Fig. 18, panel B, lanes 1, 2 and 5), and two CMPs with molecular masses of 17 and 85 kdal (Fig. 18, panel B, lane 4). The 17 kdal OMP also reacted with this antiserum (Fig. 18, panel B, lane 3). "Anti-81" serum reacted with soluble proteins of molecular masses of 85 kdal or greater, but weakly with the soluble or membrane bound 17 kdal protein (Fig. 18, panel C). The "anti 81+17" serum reacted with proteins also reactive with the "anti-17" serum, including the 17 kdal OMP (Fig. 18, panel D).

Strain MS-1 cells showed a strong reaction with the anti-17 serum whereas those incubated with normal rabbit serum or PBS (e.g.

background) reacted weakly (Fig. 19).

Discussion

Serum "anti-17" reacted with numerous soluble proteins and hemoproteins which apparently have regions of antigenic similarity shared with the 17 kdal immunogen. Reactivity of the "anti-17" serum with both the 17 and 85 kdal CMPs was expected. Spectral evidence indicated that these were membrane-bound c- and cd_1-type hemes, respectively. Reactivity of the 17 kdal OMP with "anti-17" was unexpected.

To insure that the reactivity was to cytochrome c_{551} of strain MS-1 and not to Mycobacterium proteins (2 $mg \cdot ml^{-1}$) present in Freund's complete adjuvant, this entire experiment was repeated using Freund's incomplete adjuvant. Once again, the same proteins, including the 17 kdal OMP, reacted.

Since relatively few soluble proteins reacted with antiserum raised against the 81 kdal hemoprotein, it is conceivable that this hemoprotein does not share major antigenic determinants with either the 17 kdal OMP or the 17 and 81 kdal CMPs. Interestingly, these latter proteins reacted with antiserum collected from the animal immunized with the 81+17 antiserum. This finding strenthens the hypothesis that the 17 kdal soluble heme shares antigenic determinants with other cell proteins than does the 81 kdal heme.

The reaction of whole cells with anti-17 serum suggests that the 17 K OMP is exposed on the outer surface of cells of this strain. These results corroborate those described previously with cellular

separation methods and the Western blot technique.

Although the 17 kdal OMP and CMP reacted with the "anti-17" serum, they did not show peroxidase activity. Either the treatment used to separate the outer from the cytoplasmic membrane destroyed enzymatic activity or these proteins do not possess peroxidase activity.

At least some periplasmic and OMPs are thought to be synthesized separately from those of the cytoplasmic membrane (9). Cytoplasmic proteins lack the signal peptides of periplasmic and OMPs. These peptides are apparently removed during translocation through the membrane. If this pattern of protein biosynthesis and assembly is also characteristic of A. magnetotacticum cells, then the 17 kdal periplasmic and OMP, but not the CMP of similar mass should contain signal peptides. Amino acid sequence homology analysis of the soluble 17 kdal c_{557} hemoprotein and the 17 kdal OMP are needed to determine whether these cells synthesize a hemoprotein which is localized on the outer surface. A hemoprotein in this membrane may serve to protect the cell from harmful effects of oxygen rather than possessing a role in energy transduction.

Acknowledgments

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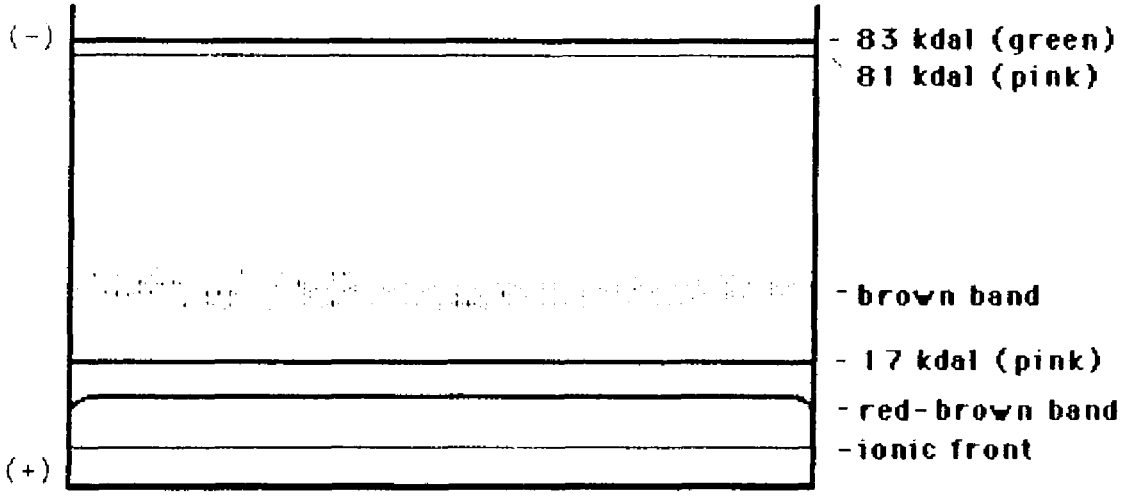

FIG. 16. Schematic representation of periplasmic proteins (12-25 mg) of Aquaspirillum magnetotacticum strain MS-1 solubilized at 25°C and separated in a preparative SDS-polyacrylamide gel. These pigmented proteins were seen on a unstained gel. The 17 and 81 kdal hemoproteins were pink and the 83 kdal was green in color.

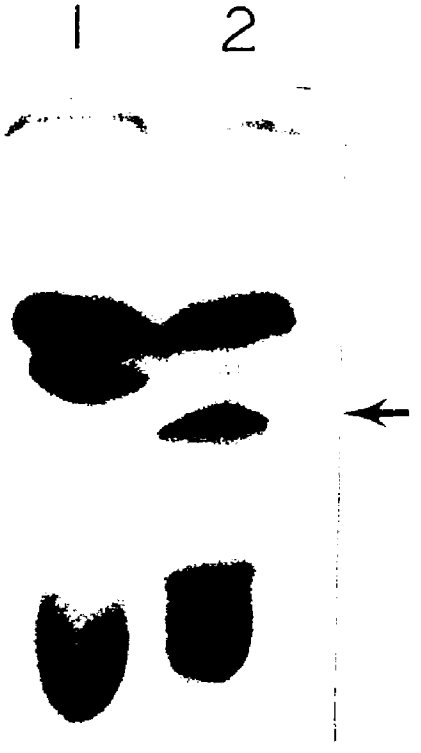

FIG. 17. Diaminobenzidine-peroxidase stain of Aquaspirillum magnetotacticum periplasmic proteins (63 µg per lane) separated on a 17 % SDS-polyacrylamide gel. Prior to electrophoresis, proteins were solubilized at either 100°C for 5 min, lane 1; or at 25°C, lane 2. The arrow indicates the position of the 17 kdal pink hemoprotein.

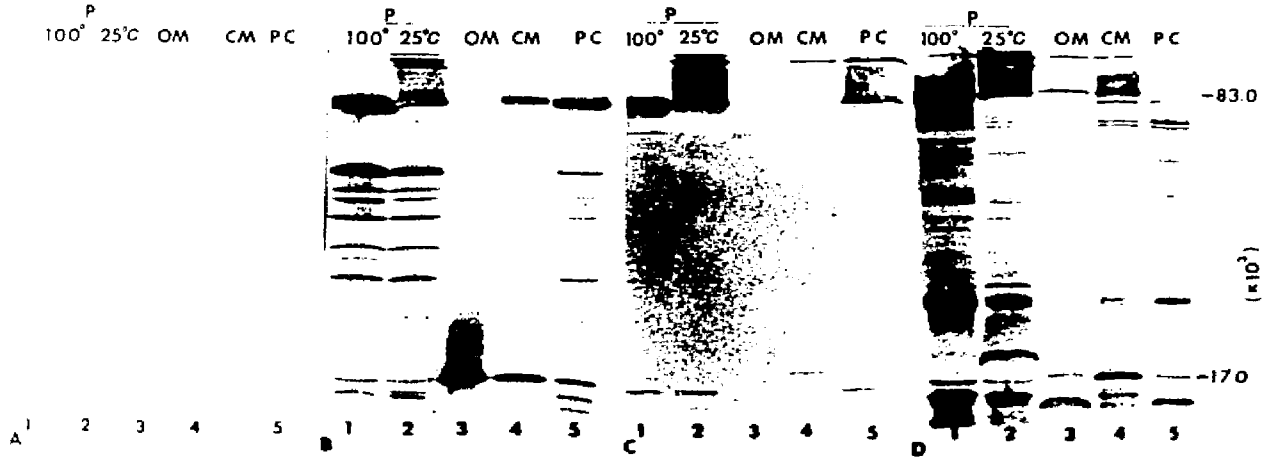

FIG. 18. Reactivity of A. magnetotacticum strain MS-1 cellular fractions with: preimmune serum, panel A; anti-17 serum, panel B; anti-81 serum, panel C and anti-17+81 serum, panel D. The positions of the 17 and 83 kdal proteins are indicated. Abbreviations: P, periplasm; OM, outer membrane; CM, cytoplasmic membrane; P/C, periplasm and cytoplasm. Periplasmic proteins were solubilized at either 25 or 100°C prior to electrophoresis. Gels used in the transfer contained 30 μg protein per lane.

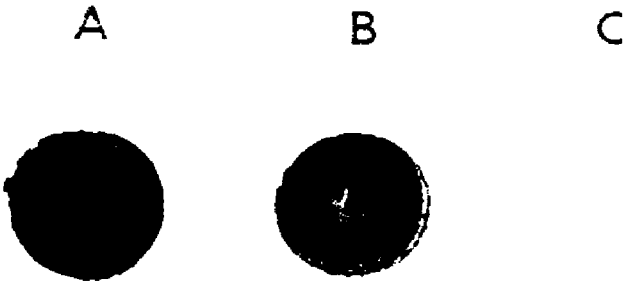

FIG. 19. Filter assay using whole A. magnetotacticum cells exposed to: anti-17 serum, row A; normal rabbit serum, row B; or PBS, row C.

CHAPTER SEVEN

Chapter Seven

Iron Relieves Nitrate Limitation in Continuous Cultures of *Aquaspirillum magnetotacticum*.

Abstract

Aquaspirillum magnetotacticum strain MS-1 cells in nitrate-limited continuous cultures did not show an increase in growth when iron was raised from 2 to 4 or from 4 to 8 μM. However, when iron was increased from 8 to 16 μM the culture biomass doubled and oxygen consumption rate per milligram of biomass increased five-fold. Further increases in biomass were not observed when iron was raised to 32 μM. The increase in biomass between 8 and 16 μM iron may represent a shift in cell respiration from nitrate to oxygen as terminal electron acceptor.

Introduction

Continuous culture methods provide benefits of precise regulation of oxygen, pH and nutrients and permit study of cells maintained at constant growth (4). This method is ideal for organisms such as microaerophilic, magnetotactic bacteria which grow best in a narrow range of pH and oxygen.

Batch cultured cells of the Gram negative organism, *Aquaspirillum magnetotacticum* strain MS-1 optimally convert extracellular ferric iron to magnetite under microaerobic (Po_2 of 0.5-1 kPa) conditions (1). Cells cultured in this manner were able to translocate protons in response to O_2, NO_3^- and Fe^{3+} (8). We became interested in the effect of iron on cell respiration with NO_3^- and O_2 as oxidants.

Studies on iron transport in A. magnetotacticum revealed that cells secrete a hydroxamate-type siderophore when cultured with high (20 μM) iron (4). Below 10 μM iron, the siderophore was not produced although a 55,000 dalton iron-repressible outer membrane protein (IROMP) was formed. These results were consistant with the possibility that this organism employs at least two modes of iron transport: one of low specificity and another, hydroxamate-mediated high affinity system. Furthermore, the average cell magnetite yield increases directly with increase culture iron (L. Paoletti, M.S. thesis, University of New Hampshire, 1984). These effects were observed using cells grown microaerobically in sealed batch cultured in which conditions vary during growth.

We undertook this work to establish the parameters necessary to grow and maintain magnetism in A. magnetotacticum strain MS-1 cells in continuous culture. We have investigated the relationships of iron concentration to growth and magnetite yield and the role of iron in cell respiration particularly with NO_3^- and O_2 as terminal oxidants.

Materials and Methods

Culture conditions. A. magnetotacticum strain MS-1 was grown in continuous culture in a two liter, water-jacketed glass vessel (Pegasus, Ontario, Canada) at 30°C. The principal carbon and energy sources were tartaric and succinic acids (3 mM each), and 0.36 mM sodium acetate. Ascorbic acid (0.2 mM) was added as a reducing agent. Iron was supplied as ferric quinate (FeQ) and unless otherwise noted, nitrate was at a growth limiting concentration (4 mM as $NaNO_3$). The

chemostat vessel was seeded by pumping sterile medium from the vessel into a 15 l carboy containing motile, magnetotactic cells. The vessel was then refilled by pumping cells and medium back into the vessel. Throughout the procedure, the vessel was continuously sparged with sterile (filtered) N_2. Culture mass doubling time (t_D) was maintained at 9.2 h with D=0.075 h^{-1}. Dissolved oxygen was monitored with a galvanic electrode (model M1016-0208, New Brunswick Scientific Co., Edison, NJ) and maintained at 1 % of saturation by adjusting the nitrogen-to-air ratio of a mixture supplied at a constant rate of 3.3 cc $\cdot$ min^{-1}. The culture pH was monitored with an autoclavable electrode (type 465, Ingold electrodes, Wilmington, MA) and was continuously maintained at a value of 6.8 by means of a pH controller (model pH40, New Brunswick Scientific Co.) delivering sterile 3 N HCl. Although the medium was not deferrated, at no time during growth did the culture or any solutions added to it come in contact with ferrous metals. The total background iron content of the medium to which no FeQ was added was 0.35 μM.

Growth measurements. Samples for dry weight measurements, cell counts, and electron microscopy were periodically collected from the outflow of the reaction vessel and fixed with 1 % formalin. Steady state samples (500 ml) were collected on ice and immediately cleared of cells by centrifugation (5,000 x g, 30 min, 5°C). Supernatant fluids were concentrated 50-fold on a rotary evaporator at 30°C and filtered through a 0.22 μm polycarbonate membrane filter. Concentrated cells and fluids were stored at -20°C for analysis. Direct cell counts were made with a Petroff-Hausser bacterial counting chamber. Biomass (dry

weight) of each sample was estimated by filtering 10 ml through a preweighed 0.22 μm membrane polycarbonate filter. Filters were dried to constant temperature in a 60°C oven for 24 h and reweighed.

Oxygen consumption. Cell oxygen consumption rates were measured using a Biological Oxygen Monitor (Yellow Springs Instrument Co,. Yellow Springs, OH). Samples of steady state culture collected from the outflow, were concentrated 4.6 fold by centrifugation (9,000 x g, 20°C, 10 min) and suspended in 3 ml of the supernatant fluid. Concentrated cell suspensions were placed in the sample chamber with a magnetic stir bar and maintained at 30 ± 0.5°C in a waterbath. The chamber was sparged with O_2-free N_2 (with the oxygen electrode partially inserted) until the headspace above the sample was 4-10 % of air saturation. With the electrode then placed in the cell suspension, the initial dissolved oxygen tension (d.o.t.) was ca. 20 % of saturation. This was a critical step as cells did not consume oxygen if the initial chamber headspace d.o.t. exceeded 40 % of saturation. Oxygen consumption rates were calculated from duplicate measurements over the range 0.5 to 18 % of saturation. Duplicate samples at each steady state were analyzed.

Electron microscopy. Cells were negatively stained with 0.5 % uranyl acetate and examined with a Hatachi 600 STEM operating in the TEM mode. Magnetosomes within each of 100 cells collected at each steady state were enumerated.

Results

With iron held constant at 8 μM, culture biomass approximately doubled with each doubling of nitrate concentration over the range 2 to 8 mM (Fig. 20). Biomass increase between 2 and 8 mM NO_3^- coincided

with a 566 % increase in cell number and a 144 % rise in optical density (O.D.) units (Fig. 20). This established that nitrate, provided as both an electron acceptor and sole source of nitrogen, was a limiting nutrient at 4 mM, the concentration used in subsequent studies with iron. This organism is diazotrophic but cells do not fix N_2 with 4 mM NO_3^- present.

Increase in iron from 2 to 4 and from 4 to 8 μM had little effect on biomass yield of nitrate-limited cells (Fig. 21). However, biomass doubled when the culture iron was increased from 8 to 16 μM. This was observed in each of three separate trials over this range of iron concentration. This unexpected result indicated that at some value between 8 and 16 μM, iron relieved cells of nitrate limitation. Additionally, biomass increases were not observed when the iron concentration was raised from 16 to 32 μM (Fig. 21).

The increase in biomass effected by raising the culture iron concentration from 8 to 16 μM iron was accompanied, within approximately 10 h, by an increased culture oxygen demand. Unless the culture d.o.t. was maintained at 1 % by providing O_2, the growth increase was not observed and the culture became non-magnetic (Fig. 21). Controlled addition of oxygen provided for resumption of growth (Fig. 22, arrow). However, cell biomass recovered to only 35 % of the expected value when microaerobic conditions were restored, and cells remained non-magnetic. This illustrated that oxygen was required for growth and that cells which experienced oxygen limitation were unable to produce magnetite.

Respiration rates for steady state cells cultured with 8 and 16 μM

FeQ were 0.93 and 4.3 ng O_2 consumed · min^{-1} · mg^{-1} dry weight biomass, respectively. Thus, although culture biomass doubled with doubling of iron concentration over this range, the O_2 consumption rate increased nearly 5-fold.

Culture fluids obtained at each steady state did not contain detectable levels of hydroxamates. Cells produced the 55,000 dalton IROMP at each iron concentration. Outer membrane protein profiles of continuously cultured cells at each iron concentration were identical to those reported by Paoletti and Blakemore (4) of batch grown cell with 0 or 5 μM added iron.

An average of 7 magnetosomes per cell were measured regardless of the iron concentration, although at lower (2 and 4 μM) iron concentration the particles were smaller than normal.

Discussion

With careful monitoring of oxygen and pH, A. magnetotacticum MS-1 cells grew and remained magnetic for over the 22 generations in continuous culture. Moreover, growth chracteristics (O.D., cell numbers, dry weight biomass) were similar for replicate continuous culture experiments. Dry weight measurements were the most representative and reliable method for determining biomass of magnetotactic cells.

Nitrate was determined to be limiting at 2 and 4 mM, the former concentration normally used in batch culture. We chose to use 4 mM NO_3 in subsequent experiments because of increased (more accurately measured) O.D. values, biomass and cell numbers.

Since iron in micromolar quantities was not merely substituting for nitrate (present in millimolar amounts) as the terminal electron acceptor, we sought another explanation for it's stimulatory role. The increase in biomass and oxygen demand triggered by the shift to 16 μM iron was consistant with a shift in cell respiration from NO_3^- to O_2 as the terminal acceptor. Thus, iron presumably relieved cells of nitrate limitation.

Although magnetosomes contained small quantities of magnetite from cells cultured with low iron (2 or 4 μM), magnetosome production did not appear to be linked to biomass increase from 8 to 16 μM FeQ. Thus, iron may have been involved with biosynthetic functions, such as hemoprotein formation (7), not involving magnetite production.

The range of iron values effecting this change in respiration was similar to that shown previously to effect siderophore production and synthesis of the IROMP by cells in batch culture (4). In batch cultured cells, the IROMP was formed at low iron when siderophore synthesis was repressed (4). This pattern persisted in chemostat cultured cells as the IROMP was formed but hydroxamates were not detected. These results indicate that information concerning siderophore, outer membrane proteins and magnetosome production (normally obtained from cells in batch cultures), may not be consistant with results obtained using continuous cultured. These processes may be growth-rate dependent and warrants further attention.

Regarded as a trace nutrient requirement, iron has an integral role in bacterial metabolism ranging from respiration to DNA synthesis. Low levels of iron has been shown to result in decreased levels of

cytochromes and enzymes involved with respiration (6). Of carbon, sulfur or iron limitation in nine different bacterial cells, the latter element most drastically reduced the content of a-, b-, c-, d-, and o-type cytochromes (2). In two cell types, Erwinia herbicola and Pseudomonas putida, iron-limited growth led to a complete loss of cytochrome c (2).

Perhaps growth in low (< 8 μM) iron also results in a decrease of respiratory components in A. magnetotacticum strain MS-1 cells and the relief of nitrate limitation by iron is associated with a switch in terminal oxidases of this organism (3). Low temperature spectral analysis of cytochromes and nitrite reductase (cytochrome cd_1) assays of the magnetic spirillum continuously cultured at 8 and 16 μM iron are currently in progress. With information gathered with these studies we expect to further our understanding of iron's role in respiration in this obligate microaerophile.

Acknowledgments

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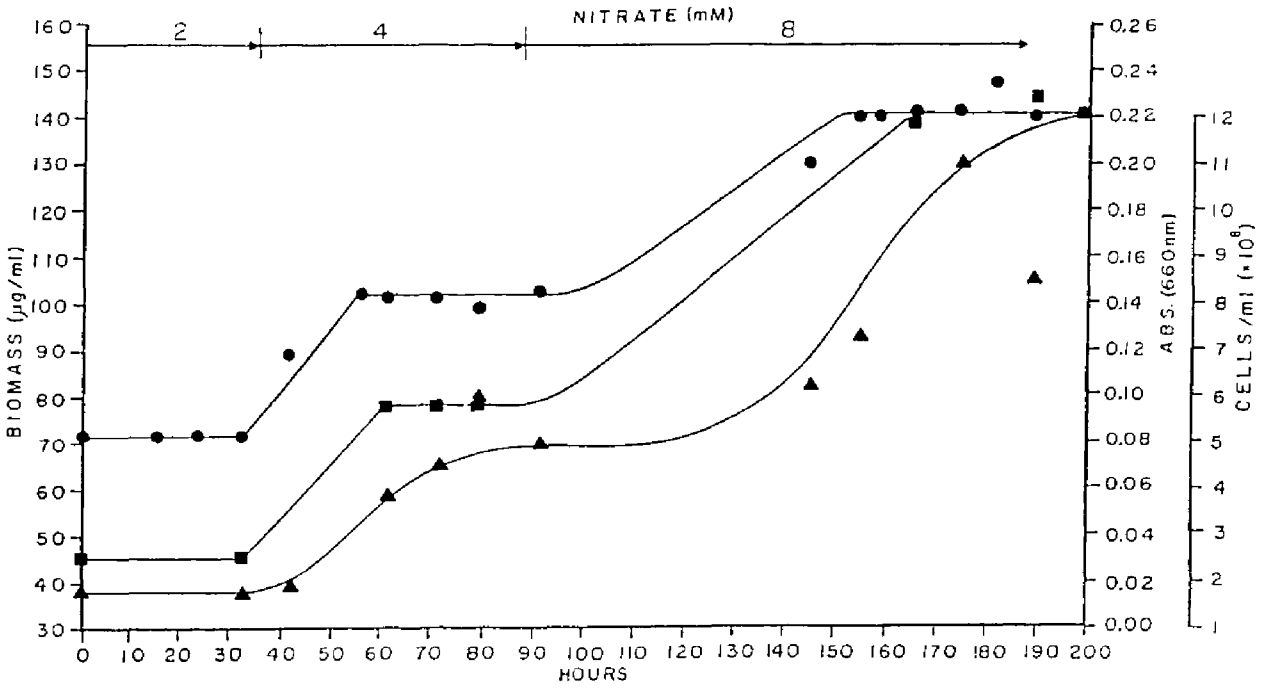


FIG. 20. Effects of nitrate concentration on growth of Aquaspirillum magneotacticum strain MS-1 with 8 µM iron. Symbols: absorbance at 660 nm, ● ; biomass, ■ ; and cell numbers, ▲ .

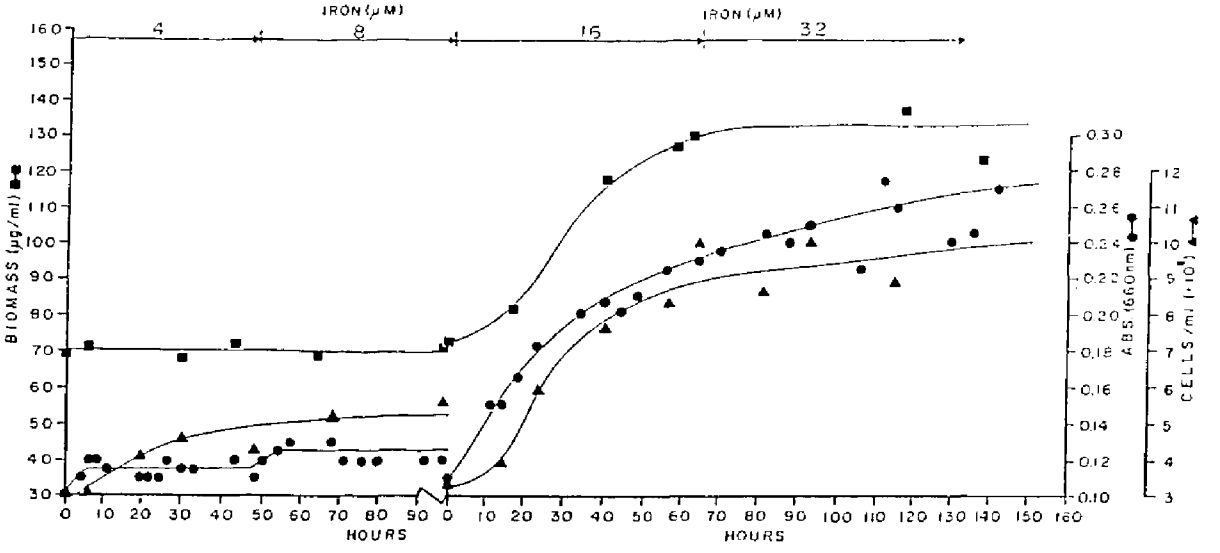


FIG. 21. Effects of iron on nitrate-limited cells of Aquaspirillum magnetotacticum strain MS-1. Symbols: absorbance at 660 nm, ● ; biomass, ■ ; and cell numbers, ▲ .

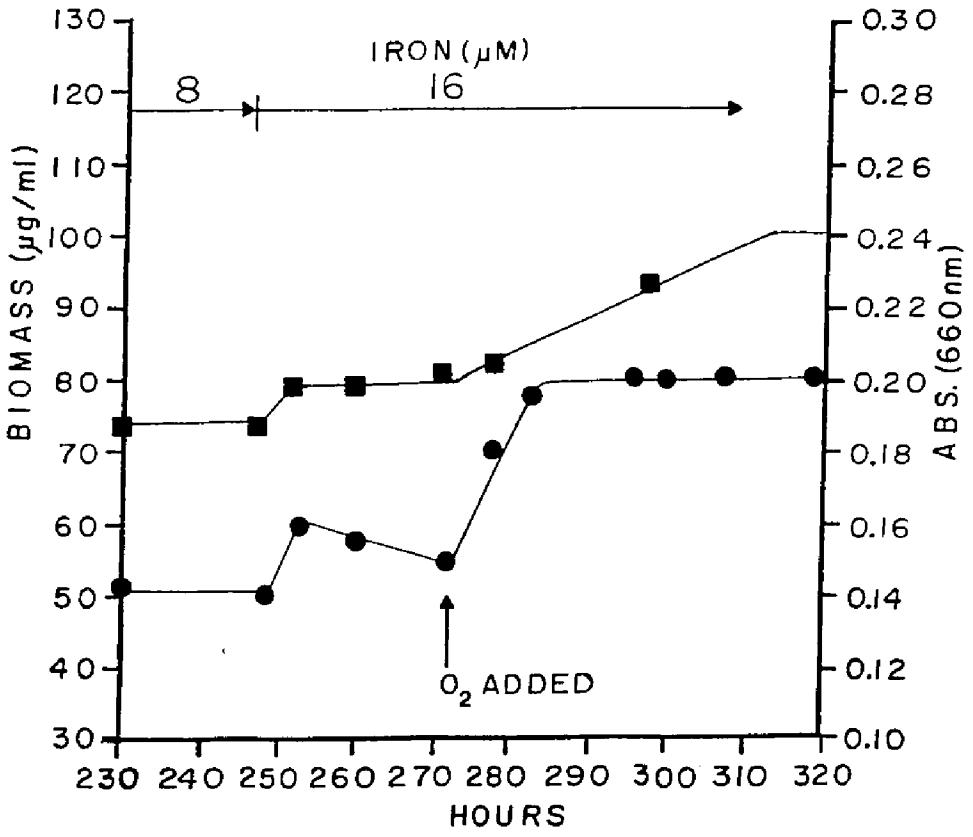


FIG. 22. Response of Aquaspirillum magnetotacticum strain MS-1 cells exposed to oxygen limitation. Arrow indicates the addition of oxygen to 1 % of saturation. Symbols: absorbance at 660 nm, ● ; biomass, ■ .

APPENDIX A

APPENDIX A

Purification of Hydroxamates Produced by

Aquaspirillum magnetotacticum.

Introduction

Most of the siderophores isolated to date have been hydroxamates. These compounds are detected in spent culture fluids with the Csaky test (2), by reaction with iron perchlorate in dilute acid, by the universal siderophore plate assay (5) or with bioassays (2). Besides catechol type chelators, all of which fluoresce, a third category of "miscellaneous"-types has been forwarded (3). In their ferric form, these chelators are not highly colored but do absorb in the ultraviolet range. Neilands predicts these compounds to be amino or imino carboxylic acids (3).

Based upon results obtained with the Csaky test and a bioassay using Salmonella typhimurium enb-7 (a mutant unable to produce chelators), A. magnetotacticum strain MS-1 has been shown to secrete hydroxamates solely when cultured with high (20 μM) iron (4). Here we attempt to isolate hydroxamate from spent culture fluids of magnetic bacteria cultured with 20 μM ferric quinate. We followed the procedure detailed by Gibson and Magrath (1) used in the isolation and purification of aerobactin, a hydroxamate produced by Aerobacter aerogenes.

Materials and Methods

Bacterial strain and growth condition: Aquaspirillum magnetotacticum strain MS-1 was grown in MSGM (4) in a 13 l glass carboy. The pH of

the medium was monitored (model pH40, New Brunswick Scientific Co.) and maintained at 6.7 with the addition of sterile 4 N nitric acid. Total amount of nitric acid added during growth was 46 ml.

Culture fluid. Cells were separated from growth fluids by tangential flow filtration using 0.45 μ membranes (Millipore Cassette System, Millipore, New Bedford, Massachusetts). Prior to use, the cell harvester was rinsed with 30 l of distilled and 10 l of Milli-Q (< 10 mOhms) water. Culture fluids (12 l) were passed at room temperature through a Dowex-1 (Cl^-) column (5 x 6 cm) at a rate of 4 $l \cdot hour^{-1}$. The column was stored overnight at 4°C. The following day, the column was eluted with 0.7 M NH_4Cl at 1 $ml \cdot min^{-1}$. Ten ml fractions were collected and aliquots tested for chelators with 0.12 M $FeCl_3$ dissolved in 0.005 N HCl solution. Approximately 2 h after the addition of NH_4Cl a brown-orange band was observed in the column above a narrow green band.

Elution profile is presented below.

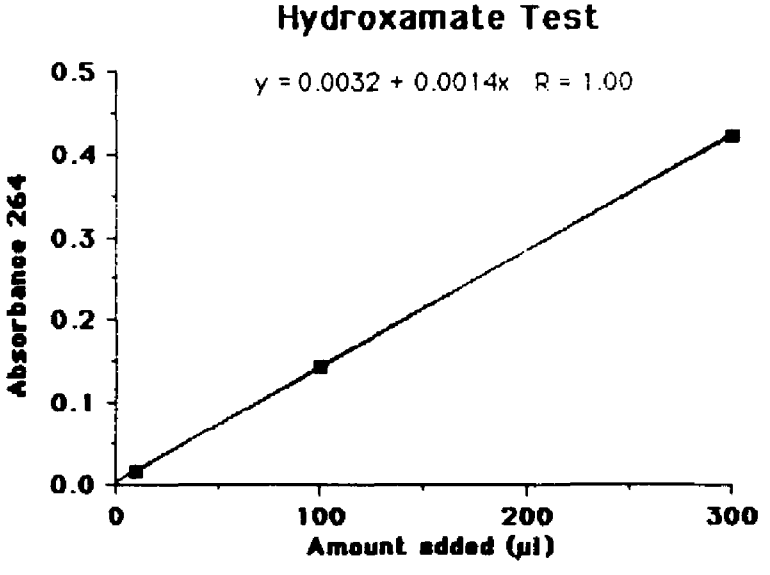
	Reaction with $FeCl_3$		
Tube number	color	precipitation	Comment
2-10	yellow	-	
11-14	yellow	+/-	
15-22	yellow	++	effluent blue
23-24	yellow	+	
25-29	orange	-	green band eluted

Tube number 19 to 29 were collected. Spectral scans revealed two peaks at 250 and 300 nm with a slight trough. One hundred ml of

this material was concentrated to 14 ml and 5 ml was placed onto a G-75 superfine Sephadex column. This column was eluted with Milli-Q water at a flow rate of 0.5 ml • min^{-1} and 5 ml fractions were collected. Out of 60 tubes collected, only tube number 9 displayed a precipitation reaction with the iron solution. No other reaction was observed. Tube numbers 8-10 were collected and rotary evaporated to dryness. Blue-green crystals (576 mg) were collected. Residual crystals were removed by a small amount of Milli-Q water and tested for hydroxamates by a modification of the Csaky reaction. Results of this test are presented below.

µl of preparation added	Optical density (264 nm)
0, Blank (subtracted)	0.08922
10	0.01616
100	0.14440
300	0.42198

These data are presented graphically below.



Using 10 µl of the above preparation as a blank and 100 µl as a test, the spectrum displayed a sharp maxima at 219 nm. Final concentrations of 0.1 to 1.0 % of this material did not reverse iron limitation in a bioassay using Salmonella typhimurium enb-7 mutant (4).

We tried to recover hydroxamates from culture fluids of strain MS-1 using a general procedure outlined by T. Stull (personal communication, Medical College of Pennsylvania). The protocol used was:

1. Adjust the pH of postgrowth culture fluids from strain MS-1 to 2.0 with perchloric acid. At this pH, a red color should develop. This color did not appear during my attempts with culture fluids from MS-1, thus the rest of the procedure was not performed.
2. Add chloroform to fluid at a 1:6 volume ratio.
3. Centrifuge to remove chloroform layer which is red in color.
4. Reextract fluid as described in step 2 and combine chloroform fractions.
5. Evaporate to dryness under a stream of nitrogen gas.
6. Suspend material in a small amount of methanol.
7. Test sample by thin layer chromotography using known chelators and iron perchlorate spray.

Literature Cited

1. **Gibson, F. and D. I. Magrath.** 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by Aerobacter aerogenes 62-I. Biochem. Biophys. Acta. **192**:175-181.
2. **Holzberg, M. and W.M. Artis.** 1983. Hydroxamate siderophore production by opportunistic and systemic fungal pathogens. Infect. Immun. **40**:1134-1139.

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4. **Paoletti, L.C. and R.P. Blakemore.** 1986. Hydroxamate production by Aquaspirillum magnetotacticum. J. Bacteriol. **167**:73-76.

5. **Schwyn, B. and J.B. Neilands.** 1986. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47-56.

AFTERWORD

AFTERWORD

Aquaspirillum magnetotacticum strain MS-1 was the first organism reported to produce hydroxamate chelators at high (20 and 40 μM) but not at low (5 μM) iron. Recently, it was shown that Haemophilus influenzae cells optimally produced hydroxamates with 100 μM iron. It would be interesting to determine the culture iron concentration necessary for optimal production by strain MS-1. Isolation and purification of this hydroxamate may then prove more feasible. Other as yet undetected chelators may be produced by these cells especially at low iron.

The possible role in iron binding and/or transport of the 55,000 dalton iron repressible outer membrane protein remains unknown. Because strain MS-1 cells grown with iron citrate produced a 58,000 dalton outer membrane protein, not otherwise seen with cells provided with iron quinate, a citrate-dependent iron uptake system may also exist. These possibilities have not been throughly investigated.

A working model based upon currently available information for iron uptake by strain MS-1 is presented in Figure 23. The references presented are cited in preceding literature reviews.

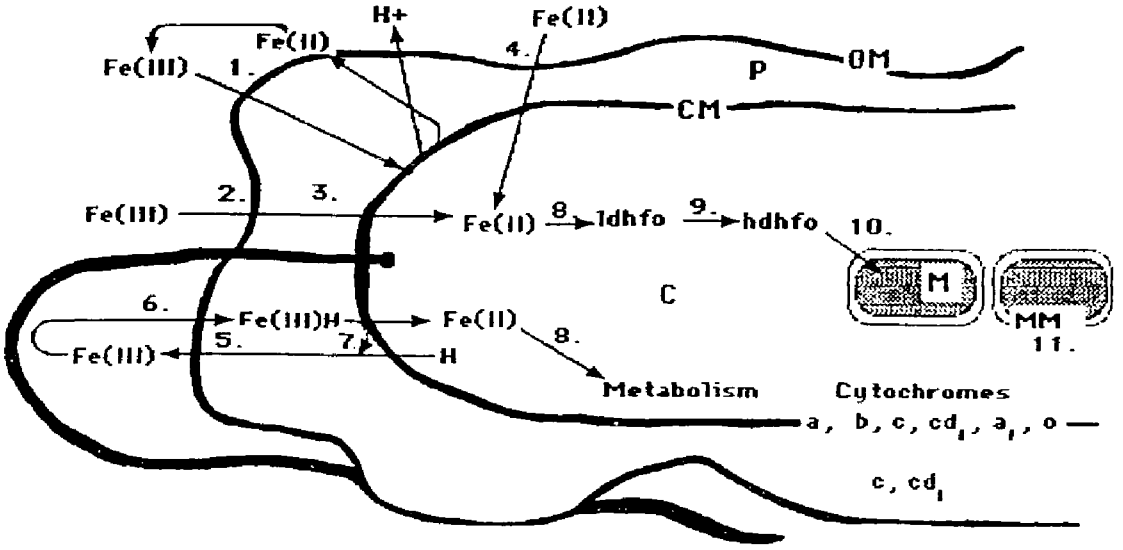

FIG. 23. Proposed mechanism for iron transport by Aquaspirillum magnetotacticum strain MS-1. Abbreviations: OM, outer membrane; P, periplasm; CM, cytoplasmic membrane; C, cytoplasm; M, magnetosome; MM, magnetosome membrane; H, hydroxamate; ldhfo, low density hydrous ferric; hdhfo, high density hydrous ferric oxide.

Site	Process	Reference
1.	Protons are translocated in response to iron as the sole oxidant.	Short and Blakemore, 1986
2.	Ferric iron uptake occurs via energy-dependent active transport (Km = 35 μM and Vmax = 1.25 nmol/min/mg biomass).	Paoletti and Blakemore, unpub. results.
3.	Most (77 %) of iron reductases recovered was in the cell periplasm.	Paoletti and Blakemore, 1988
4.	Ferrous iron was also inwardly transported by cells but at rate 133 % less than for ferric iron.	Paoletti and Blakemore, unpub. results.
5.	Hydroxamate chelators were detected in culture supernatant fluids from cells grown with 20 and 40 but not with 5 μM iron.	Paoletti and Blakemore, 1986
6.	Hydroxamate chelator shown to be a siderophore as denoted with a bioassay and Salmonella typhimurium enb mutant.	Paoletti and Blakemore, 1986
7.	Mechanisms of ferric-hydroxamate reduction by this organism are currently not known.	
8.	Internalized iron is either processed into magnetite or used for other aspects of metabolism, such as in the formation of cytochromes (some of which are soluble).	O'Brien et al., 1987
	Ferrous iron undergoes a reoxidation to form a low density hydrous ferric oxide.	Frankel et al., 1983
9.	This is followed by a dehydration to form a high density hydrous ferric oxide also called ferrihydrite.	Frankel et al., 1983
10.	Ferrihydrite undergoes further dehydration and reduction to form magnetite (Fe_3O_4).	Frankel et al., 1983
11.	Magnetosome membranes contain proteins unique to it. These may serve a role in the compartmentalization of iron in these cells.	Gorby et al., 1988